UNIVERSITA' DEGLI STUDI
DI GENOVA

DISTAV

Department of Earth, Environment and Life Sciences

PhD thesis:

Population dynamics and structure of sperm whale (*Physeter macrocephalus*) in Mediterranean Sea

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PhD in Marine Sciences

2020

Ai miei genitori,
mia forza, mio pilastro,
mia fonte di energia
nel coltivare
ogni mia passione

"Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza…"
Ulisse - Inferno, canto XXVI, vv. 118-120
Dante Alighieri

"when you set out for Ithaca
hope the voyage is a long one.
Laistrygonians, Cyclops, angry Poseidon
don't be afraid of them…"
Ithaka
C. P. Cavafy

Old drawing of Hercules while placing his pillars in Gibraltar Strait

Acknowledgements

I need to underline that this project would not have been possible without:

The precious inspiration, support and help of Prof. Alberta Mandich, prof. Maurizio Würtz and Prof Rus Hoelzel.

The grants provided by Genoa University and Ocean Care foundation.

My wonderful working teams of Menkab, il respiro del mare and Artescienza.

The fieldwork experiences with Menkab, il respiro del mare association in Ligurian Sea, Futurismo Azores whale Watching in Azores, Moidjio CRCAD in Comoros

The provided samples by Frantzis A[4], Alexiadou P[4], Ody D[5], Tardy C.[5], de Stephanis R[6], Giménez J[7], Bittau L[8], Leone M[8], Camedda A[9,] de Lucia G[9], Lucifora G[10], Lopez A[11,12], Covelo P[11,12], Alves F[13], Dinis A[13], Tejedor ML[14], Fernández A[15], Arregui M[15], Arbelo M[15], Silva M[16], Oliveira C[16] (affiliations [4]Pelagos Cetacean Research Institute, Vouliagmeni, Greece; [5]WWF France, Marseille, France; [6]CIRCE, Conservation Information and Research on Cetaceans, Cadiz, Spain; [7]ICM-CSIC,Institut de Ciències del Mar Barcelona, Spain; [8]SEAME Sardinia Onlus, La Maddalena, Italy; [9]IAS-CNR Institute of Anthropic Impact and Sustainability in marine Environment, Oristano, Italy; [10]Istituto Zooprofilattico Sperimentale del Mezzogiorno; [11]Departamento de Biologia, Universidade de Aveiro, Portugal; [12]CEMMA, Coordinadora para o Estudo dos Mamíferos Mariños, Galicia, Spain; [13]MARE - Marine and Environmental Sciences Centre / ARDITI, Madeira, Portugal; [14]Oriental Canary Islands Strandings Cetaceans Network, Lanzarote, Spain; [15]Atlantic Cetacean Research Center, Institute of Animal Health, University of Las Palmas de Gran Canaria, Spain; [16]IMAR - Instituto do Mar & OKEANOS – University of the Azores, Faial, Portugal; [17]Artescienza s.a.s., Varazze, Italy; and Banca dei tessuti dei mammiferi marini of Padua University).

The laboratory helps and advices received by Menno de Jong, Daniel Moore, Mario Erandi Bonillas, Vania Fonseca and prof. Rus Hoelzel.

The fundamental bioinformatic support by Menno de Jong, Daniel Moore from Durham University.

The suggestions by several colleagues in results interpretation and discussion: Fulvio Garibaldi, Philip Morin, Alan Alexandre, Andrea Moura.

The precious corrections and suggestions of my two reviewers: Alexandros Frantzis and Andreanna Welch.

The support of wonderful friends from all the Universities, Perugia, Genova and Durham, where I studied, and from all the wonderful places, Azores and Comoros, where I improved my skills and knowledge on cetaceans and where I lived unforgettable moments.

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"There she blows!
A hump like a snow-hill!
It is Moby Dick!"

Moby Dick, or the whale
Herman Melville

ABSTRACT

The sperm whale (*Physeter macrocephalus*) has a cosmopolitan distribution. In the Mediterranean, sperm whale sub-population is classified as 'Endangered' according to the International Union for the Conservation of Nature. Even though this population hasn't faced the whaling age, it is currently threatened by other anthropogenic impacts, which cause direct mortality and strandings (i.e. ship strikes, debris ingestion, entanglement in driftnets), or may cause indirect mortality (e.g. noise pollution). Previous genetic, acoustic and mark-recapture studies have indicated that this population is distinct from the North Atlantic population. Within the Mediterranean Sea, the population structure is however unclear. Photo-ID data have shown three recaptures between the eastern and the western basins, but no confirmation of breeding success between those basins is available to date. Genetic differentiation within the Mediterranean Sea has already been confirmed for various dolphin and fish species. Here, we investigate population structure and pattern of gene flow within the Mediterranean Sea, and between the Mediterranean and the close Atlantic area, using 11537 single nucleotides polymorphism sites (SNPs) generated by restriction-site-associated DNA sequencing (RADseq). We have sequenced 142 samples from stranded and free-ranging individuals, 104 samples from the Mediterranean Sea and 38 samples from the eastern North Atlantic. Results on genetic structure revealed, as suggested in previous studies, a division between Mediterranean and Atlantic areas. Through Gibraltar Straits gene flow rates are low but significant, revealing that some individuals cross the straits for breeding purposes. Within the Mediterranean, no division between western and eastern basins was found, confirming that Sicilian waters are not barriers

to both movements and the gene flow. Furthermore, we found two different populations, a first one composed of samples from different areas of the Mediterranean basins, and a second one composed of samples collected in Ligurian Sea, with both Mediterranean and Atlantic ancestries, revealing a hybrid population never described before. This latter unexpected result is the first evidence of some breeding success from Atlantic sperm whale in the Mediterranean, which may prevent the loss of genetic diversity and inbreeding process in this threatened population. Sperm whale can probably enter in the Mediterranean for exploring, looking for food and eventually mate.

The discussed results significantly improve our knowledge of the pattern of connectivity for Mediterranean sperm whales. Therefore, they are essential for developing a more effective conservation management strategy for this endangered population.

Prologue

The field of population genetics comprises a rich theoretical framework and a powerful set of analytical tools to understand how mutation, selection, gene flow and genetic drift affect patterns of populations (Allendorf *et al.*, 2010). Species with wider latitudinal distribution range face different environmental conditions, contain genetically distinct subpopulations - because of varying selection pressures and genetic drift - and show higher diversity than species with smaller latitudinal ranges (Ralph & Coop, 2010). In conservation biology, the identification and the protection of genetically distinct local populations that might have distinct gene pool and local adaptations are crucial factors for maximizing evolutionary potential and reducing extinction risks (Avise *et al.*, 2009; Hilborn *et al.*, 2003). The interplay of natural selection and genetic drift, influenced by geographic isolation, mating systems and population size, determines patterns of genetic diversity within species and populations. The local population is considered a *functional unit* in ecosystems and for this reason, biologists and managers should be able to define the geographic boundaries in order (a) to develop effective conservation plans and (b) to avoid overharvesting and pressures in that area (Luck *et al.*, 2003). The estimation of population parameters such as effective population size (*Ne*) (that is the number of individuals needed to avoid the loss of genetic diversity and inbreeding phenomena) and migration rates between subpopulations is crucial for many ecological studies, and two very different approaches are in use: direct methods using direct observations (including photo-identification techniques) or satellite-tag tracking data of migrating individuals, and indirect methods using genetic data from samples of individuals in several subpopulations for the inference of migration rates. Obviously, direct methods can help to determine the migration pattern of

individuals during the study, and can deliver information about very recent history, but if the study is too short and not repeated we cannot know if the migration pattern we observed was accidental or is general (Beerli, 1998). On the other hand, molecular markers can inform about genetic migration (successful reproduction at the new location), and provide a robust method for testing hypotheses about the evolution of population structure, population dynamics and patterns of connectivity (e.g. Avise, 2000; Hewitt, 2000; Bunje, 2005; Pauls *et al.* 2006). While numerous studies have provided useful data on phylogeography based on mtDNA sequences (e.g. Avise, 2000; Taberlet and Bouvet 1994; Frohlich *et al.* 1999; Emerson *et al.* 2000; Drouot *et al.*, 2004; Gaspari *et al.*, 2013) and sometimes together with nuclear microsatellite DNA markers (e.g. Burton *et al.*,2002; Fijarczyk *et al.*, 2011; Theissinger *et al.*, 2013; Gaspari *et al.*, 2007), next generation sequencing methods can now greatly increase the resolution of population genetic analyses at a similar or lower cost (e.g., Godinho *et al.* 2008; Brito and Edwards 2009; Alexander *et al.*, 2012). With the emergence of these novel high-throughput sequencing methods, it is now possible to study genetic variation across whole genomes, and 'genome sampling' methods (such as Restriction Associated DNA: RAD sequencing "ddRAD-seq" Baird *et al.* 2008) can be used to identify thousands of single nucleotide polymorphism (SNP) loci at the same time as individuals are being genotyped at those loci. SNP markers, reproducible across technologies and laboratories, are ideal for long-term studies of globally distributed species such as sperm whales (*Physeter macrocephalus* = *P. catodon,* Linneo 1758), a species of conservation concern because of both historical and contemporary impacts, worldwide and at regional level.

The sperm whale provides an interesting example of a long-lived species with few geographic barriers to dispersal. This species is literally found "from pole to pole" (see Whitehead, 2003). Several studies demonstrated that apex predators, such as the sperm whale, play important roles in the workings of natural ecosystems (Smith *et al.*, 2013), in the balance of the oceanic ecosystems (i.e. Würtz and Simard, 2007) and are considered as effective indicators of marine ecosystem health (e.g. the Marine Strategy Framework

Directive 2008/56/CE uses cetaceans, among other features, to evaluate good environmental status in the European Seas). Recently, new findings on the ecology of the sperm whale have highlighted the importance of this species in the struggle for the conservation of marine ecosystems. Indeed, great whales contribute to primary production through the vertical mixing and recycling of Carbon and limiting nutrients in the ocean (Roman and McCarthy 2010). Sperm whales feeding on deep-living prey and defecating at the surface, facilitate the upward transport of iron to the photic zone. Nicol *et al.*, (2010) demonstrated that in producing blubber, whales assimilate little of their dietary iron, and their faecal plumes have an iron concentration at least 10 million times greater than ambient levels: the resulting new primary production could result in the export of at least 200,000 tons of carbon dioxide per year from the atmosphere to the deep ocean (Lavery *et al.*, 2010). Furthermore, whale carcasses sequester carbon to the deep sea, where they provide habitat and food for many endemic invertebrates (Roman *et al.*, 2014).

Sperm whale drawing
(©Maurizio Würtz – Artescienza)

The population genetics of sperm whales has been investigated world-wide, and there are several key features in the resulting findings. First, genetic variation is low especially at mtDNA (even for whole mitochondrial genomes;

Alexander *et al.* 2012; 2017), and low enough to suggest a historical population bottleneck (see Lyrholm & Gyllensten 1998) or cultural hitchhiking (see Whitehead 1996, 1998, 2003). Second, while there is evidence for female philopatry (population structure within ocean basins at mtDNA markers), nuclear markers suggest greater gene flow among regions mediated by males (Engelhaupt *et al.* 2009, Mesnick *et al.* 2011). Third, two available studies have shown that the Mediterranean basin seems to be isolated for both female and male mediated gene flow (Drouot *et al.* 2004, Engelhaupt *et al.* 2009) – even if many questions are still open. To date there have been no studies assessing population genetics "strictly" within the Mediterranean Sea. Subdivision within this isolated population, classified as *Endangered* (according to the IUCN Red list), would be essential information in support of effective conservation strategies (see Notarbartolo di Sciara *et al.*, 2012). Although Frantzis *et al.* (2011) report some interesting evidence for movement between the western and eastern Mediterranean basins, there is so far no confirmed evidence of successful inter-basin reproduction.

Sperm whale calf in Ligurian Sea
(©Biagio Violi – Menkab, il respiro del mare)

In this framework, this PhD project aims in filling the gap of knowledge about the biology and ecology of the sperm whale in the Mediterranean Sea and close Atlantic areas. To date there have been no other genetic studies within Mediterranean Sea (1) using as many samples as we did in this project, (2) covering all the main areas used by sperm whales and (3) using Next generation sequencing methodology. This is the first study focused on the Mediterranean sperm whale population, and the first study that apply RADseq protocol to this species.

The presented genetic results add important pieces to the complex puzzle of the biology of one of the most fascinating predators of our seas. Its social structure is definitely one of the most difficult to understand and to describe. For this reason, all these new findings need to be coupled with further projects using other methodologies (such as photoID and acoustics) in order to understand even further in depth this precious and threatened species.

Sperm whale eye
(© Biagio Violi – Menkab, il respiro del mare)

1 Introduction

Sperm whales are animals of extremes (Whitehead, 2018). They are the biggest living predator of the oceans, known also by the famous *Moby Dick* (Melville, 1851). Except for humans and killer whales (Ford, 2009), few animals on Earth are as widely distributed as the sperm whale. They can be seen near the ice-edge in both hemispheres and are also common along the equator, especially in the Pacific (Whitehead, 2018). They are truly unique in their appearance with an enormous head that comprises approximately one-third of their total body length (fig. 1.0.1). Sperm whales possess two quite distinct behavioural modes: foraging and social/resting (Whitehead and Weilgart, 1991). When foraging, the animals make repeated deep dives. Modal dives are to about 600 m and for about 45 min, but dives can be much deeper (to over 1000 m), shallower (e.g., when in shelf waters 200 m deep), and/or longer. Between dives the whales come to the surface to breathe for about 9-10 min. The dive is usually signalled by the raising of flukes out of the water. The descent to depth, as well as the return to the surface, can be nearly vertical (Watwood *et al.*, 2006). In most areas of the world the primary food of sperm whales appears to be meso and bathypelagic cephalopods (squid) with mantle lengths of 0.2 to 1 m in length (Clarke 1966; Clarke 1980). However, the consumption of fish has been observed and may be an important part of the sperm whales' diet in the North Atlantic, North Pacific and off New Zealand (Kawakami 1980; Rice 1989).

In the last 3 centuries, sperm whales were the focus of two intensive hunting periods. Both 'open boat' and 'modem mechanized' whaling eras were focused on sperm whales to provide sought after oil to lubricate machine parts and light lamps, in addition to harvesting ambergris (a substance similar to

wax that can be found in the intestine) which was used as a fixative in perfumes and spermaceti oil for fine candles.

Figure 1.0.1.1.1: adult male sighted in Pico - Azores
(©Biagio Violi – Futurismo Azores Whale watching)

Whalers, after realising that the large baleen whale stocks were depleted in the early 1960s, improved and mechanized whaling activity, so that sperm whales and several baleens whales species were taken at a rate of over 20,000 per year (Best 1983). Furthermore, whalers discovered and described areas, called "grounds", where sperm whales were in high concentration (Townsend 1935). These grounds were the primary targets during recent whaling operations. Whales concentrations may be associated with steep underwater topography, high productivity and oceanographic fronts such as cold hotspot (Jaquet and Whitehead 1996; Biggs *et al.* 2000), that is, the perfect habitat for sperm whales' preys. Recent post-whaling estimates performed by Whitehead (2002) place the current numbers of sperm whales around the globe at approximately 360000, despite a previous whaling global abundance estimate of nearly 1200000 (see Evans 1987; Rice 1989; Berta and Sumich 1999).

Today, movement of males is poorly documented worldwide, and little is known on the frequency, duration, or geographical extent of these migrations (Whitehead, 2003). Sperm whales are the most social of the great whales, with adult females and sub-adults of both sexes associating in social

groups and long-term units, while adult males appear to rove over large distances (in some cases between oceans (Ivashin 1981)) on their own (Best 1979; Rice 1989; Whitehead 1993; Whitehead and Weilgart 2000). Mixed groups primarily inhabit low-latitude waters, while sub-adult males are believed to disperse from their natal groups and tend to inhabit more polar latitudes as they age and grow (Best 1979; Rice 1989). Large sexually and physically mature males return to lower latitudes to breed with females (Best 1979, Whitehead 1993; Christal and Whitehead 1997; Whitehead and Weilgart 2000). Off the Galapagos Islands, mark-recapture techniques allowed to classify different types of sperm whale associations among females and their offspring, according to their duration (Whitehead & Kahn, 1992):

- "units", an association of individuals over several years, including approximately 13 animals);
- "groups", an association of units for several days, including about 23 animals on average,
- "aggregations", a temporary association of groups, including 43 whales.

The above classifications and the average number of animals on them differ depending on the ocean and region examined (Whitehead & Kahn, 1992).

In the oceans, sperm whales display a marked sexual dimorphism, with males attaining an average length of 16 m compared with an average of 11 m in females (Rice, 1989). According to body length and age, sperm whales can be also organized in:

- "*breeding schools*", that includes females (sexually mature at 8.3-9.2m long) and their offspring of both sexes.
- "*bachelor schools*", that include pubertal (8.7-10.3m long) to sexually mature males (11-12 m long and over) and consist of loose aggregations of similar-sized whales, rarely close kin related (Lettevall *et al.*, 2002).
- "*Solitary male*", physically mature (over 14-16m), generally observed alone.

Indeed, males leave the breeding school to join the bachelor schools as they approach sexual maturity. The oceanic distribution of sperm whales varies according to the sex and age composition of the groups but may be

determined also by the food sources and suitable conditions for breeding. Females apparently disperse less than males at both the social and geographical levels. Although female sperm whales are capable of moving large distances (some records of 4000 km are in Whitehead *et al.*, 2008), factors such as foraging success, predator avoidance and social cohesion may all contribute towards the fact that, often, average home ranges span approximately 2200 km in any direction (Whitehead *et al.* 2008).

Figure 1.0.1.1.2: subadult sperm whale
(©Biagio Violi – Futurismo Azores Whale watching)

Discovery tags (deployed and recovered by whalers in the North Atlantic Ocean) have shown extreme cases of one male's longitudinal movement of 4300 km from the western to the eastern North Atlantic Ocean (Mitchell 1975) and another male's latitudinal movement of 7400 km across the equator from North Africa to South Africa (Ivashin 1967). The restriction of groups of females and immatures to low latitudes may be related to the energetic constraints imposed on females by the combination of deep diving, pregnancy and lactation. Calves need to develop diving ability before they can be weaned, and the fatty spermaceti organ may be energetically expensive. Thus, calf development may be particularly demanding on female sperm whales, as indicated by a prolonged lactation period (Best *et al.* 1984). In addition, calf thermoregulatory limitations may prevent these groups from reaching high

latitudes. It has also been suggested that prey species at high latitudes may occur too deeply for females and juveniles (Best 1979). Males may have been selected to disperse widely to productive high latitudes in order to avoid competition from females and to increase the rate of growth to maturation and breeding status. Thus, these factors may ultimately have contributed to the observed contrasting mitochondrial and nuclear genetic differentiation on a global scale.

1.1 Mediterranean population

In Mediterranean Sea, the sperm whale is one of the resident cetaceans species. This predator is widely distributed, in both the Eastern and Western basins (Gannier *et al.*, 2002; Frantzis *et al.*, 2003; Notarbartolo *et al.*, 2006). The International Union for the Conservation of Nature (IUCN, Red list) has classified the Mediterranean sperm whale as a subpopulation, with distinct conservation status from the neighbour oceanic population: Endangered (Notarbartolo di Sciara *et al.*, 2012). Despite the absence of an intense whaling activity within the area, this population is facing three main anthropogenic impacts: ship-strikes, ingestion of plastic debris, entanglement.

The social distribution and movement pattern of sperm whales within the Mediterranean Sea might be more complex than a segregation of males in the northern part, performing migrations to join breeding grounds in southern latitudes as documented for oceanic populations. As will be discussed in detail in the following paragraphs, although mostly males are observed in the northern west Mediterranean in summertime (Drouot *et al.*, 2004), some cohabit with groups of females in the southern regions, as it seems to be the case of the Hellenic Trench (Frantzis *et al.*, 2003; 2014). Indeed, in the northern Mediterranean Sea in the summertime, sperm whales are generally observed in loose aggregations, rarely forming cohesive groups at the surface, while in the southern regions they tend to form clusters of up to fifteen animals, generally including calves (Frantzis *et al.*, 2014). Consistent sightings of calves in different regions such as the Tyrrhenian Sea, the Hellenic Trench and the Balearics, strongly suggest that reproductive and breeding activity occurs within the southern part of the basin (south of 41° latitude, as suggested by Drouot *et al.*, 2004). In the Hellenic Trench new born observations indicate a mid-summer calving season (Frantzis *et al.* 2014). However, the timing of the mating season in the area is still unknown and a real migration pattern of sperm whale within the Mediterranean basin has never been documented (Rendel and Frantzis, 2016). Moreover, It is important to underline that groups of females are not strictly found in southern regions: large schools including

calves have been reported in December 2001 by Moulins & Würtz (2005), in December 2003 by Laran & Gannier (2006) and in October 2017 by Calogero *et al.* (2019) within Ligurian Sea (fig. 1.1.1), although such observations are scarce.

Figure 1.1.1: group of 2 adult female and 1 calf sighted in Ligurian Sea. (©Biagio Violi – Menkab, il respiro del mare)

In the Strait of Gibraltar area sperm whales are regularly seen on both sides of the straits (De Stephanis *et al.*, 2008), therefore, movements and geneflow – marked or low – cannot be excluded. Photo-identification has revealed that animals sighted in the Strait are regularly observed in other regions of the Western Mediterranean, right up to the northeast corner of the Ligurian Sea. Here is listed, a detailed summary on sperm whale movements described through photoID technique:

- Matthews *et al.*, (2001) documented a match between the straits and 1800 Km to the west, in Azores.
- Drouot *et al.* (2007), in the western Mediterranean, including the Ligurian Sea, the Gulf of Lions and waters off the Balearics identified 44 individuals. Of these, 11 identified whales were photographically re-captured. Analyses, within-year, revealed that re-sightings occurred from 1 to 29 d apart, while five identified whales were resighted over several summer seasons, from 1 to 7 years after. Four whales were seen in more than one year in the northern part of the basin, indicating site fidelity to this feeding area.
- Drouot & Gannier (2007), combining photo-identification and acoustic data demonstrate a north–south movement of some sexually mature males

(around 13 m in length), feeding in the northern regions and joining groups of females off the Balearics for around 20 d. These displacements ranged over ~500 km, with travelling time of seven days or less.

- Boisseau *et al.* (2010) conducted visual and acoustic surveys in the adjacent Atlantic Ocean and reported single sightings of sperm whales in Moroccan waters to the south, and Spanish waters to the north, of the Strait, and none directly west, but survey effort was too low to draw robust conclusions.
- Carpinelli *et al.* (2014), of 47 animals identified in the Strait from 1999 to 2011, 15% were identified in other parts of the western Basin between 1994 and 2011.
- Lisa Steiner in a personal communication (August 2016) said that no matches between Mediterranean and Azores catalogues have been found.

Estimating cetacean population trends is notoriously difficult even for the best studied populations (Wilson *et al.*, 1999). Several studies and projects within the Mediterranean provided abundance estimates. However, it is still not known exactly how many sperm whales are in our basin and a few regions remain still unsurveyed. Here, a summary of some abundance assessment within two Mediterranean basins:

- In the Eastern basin. Lewis *et al.* (2007) estimated 62 individuals in the Ionian Sea. Frantzis *et al.*, (2014) identified 181 individuals, after a long research program conducted between 1998 and 2009 along the Hellenic Trench. The high rate of photographic recapture and matching between the Aegean Sea and the Hellenic Trench, suggests an estimation of no more than 250 individuals (Frantzis *et al.*, 2014) and a high concentration of sperm whales along the Hellenic Trench (Boisseau *et al.*, 2010; Lewis *et al.*, 2007). Lastly, Lewis *et al.* (2018) estimate of 147 for the surveyed areas with a 95% confidence interval of 74–289. Extrapolation to unsurveyed areas suggested a total of 164 but with several caveats. In conclusion, Frantzis *et al.* (2019) suggest that all this evidence leads to the

assumption of a total population of roughly 200–300 individuals for both the Hellenic Trench and the entire eastern Mediterranean basin.

- In the Western basin. Pace *et al.* (2014) count 60 individuals around the island of Ischia after 9 years of photo-ID effort from a survey area approximately 8800 km², although the discovery curve showed no sign of a decreasing rate of new identifications, so this site may be part of a much larger home range for the western Basin population. Rendell *et al.* (2014) identified 180 individuals across the northern part of the western Basin between 1990 and 2008 and, using a variety of analytical approaches, got that none of the upper confidence bounds on the estimates exceeded 1000 individuals once sampling bias was taken into account, and lower bounds were less than 200, suggesting that the population of this area counts around 400 individuals.

These are the best data on sperm whale population abundance that we can have up to now. Potentially, these values reveal that Mediterranean sperm whale population is very small. However, none of the datasets are perfect, because restricted in time, spatial coverage, unevenness of sampling effort (Rendell and Frantzis, 2016). In conclusion, it is largely consistent with previous expert judgements that the population in the whole Mediterranean may be around the very few thousands and these data certainly support the classification of this population as 'Endangered'. Furthermore, the survey data from both line-transect and photo-identification studies are currently insufficient to answer the isolation question with confidence (Rendell and Frantzis, 2016).

Recruitment of young is a crucial factor in the population dynamics of any mammal, but here are only the most superficial observations for Mediterranean sperm whales. There is some evidence that sperm whales in the eastern Basin have a reasonable calving success, as Frantzis *et al.* (2014) reported: 15 of the 16 social units they observed between 1998 and 2009 had a calf with them at least once in that period, and that 79% of encounters with social groups featured calves. However, it is difficult to draw strong

conclusions, since calf presence does not necessarily lead to recruitment into the adult population, and the same study also reported that calf and juvenile mortality was likely to be high (>40% and >27%, respectively). Thus, to date, we remain woefully lacking in hard data on what is happening to the Mediterranean sperm whale population (Rendell and Frantzis, 2016).

1.2 Habitat preference

In Mediterranean Sea, several studies confirmed that sperm whale distribution depends on topographic and oceanographic features (see Frantzis *et al.*, 2014; Gannier and Praca, 2007; Gannier *et al.*, 2002; Pirotta *et al.*, 2011; Praca and Gannier, 2008; Praca *et al.*, 2009). On the western basin, Praca and Gannier (2008) used combined visual and acoustic surveys to show that sperm whales have a strong preference for shelf waters both along the French and Italian coasts, as well as those along the eastern Balearics; this also appears to be true of the shelf waters off southern Spain (Canadas *et al.*, 2002). Similarly, a strong association between sperm whale distribution and submarine canyons has been found in the northern Ligurian Sea (Tepsich *et al.*, 2014). Zones of high density also include the waters where depths reach 1000 m in the south of Mallorca and Ibiza in the Balearic Islands (Pirotta *et al.*, 2011). A strong association between sperm whales and a bathymetric feature called Cuma Canyon, which is less than 20 km wide, has been confirmed in the northwest of the island of Ischia just outside the Bay of Naples in Italy (Mussi *et al.*, 2014). In the eastern basin, the Hellenic Trench running from the west of the Ionian Islands to the west and south of Crete and south and east of Rhodes Island, is the home of sperm whales and have a strong and clear density peak around the 1000 m depth contour which drops off rapidly as the water gets either shallower or deeper either side of the contour (Frantzis *et al.*, 2014). In the Turkish waters, most sperm whale sightings are concentrated in the Fethiye Canyon, one of the deepest parts of the Mediterranean Sea at 4500 m (Ozturk *et al.*, 2013). All the listed studies underline the key role of physical oceanographic factors, such us current interactions with bathymetry, which have profound effects on the spatial ecology of squids, even if the exact process is still unknown. Praca and Gannier (2008) showed that sperm whale distribution in the western Basin is linked to waters with lower surface temperatures (perhaps indicating relatively recent upwelling) and higher chlorophyll (consistent with productivity boosts resulting from the upwelling of nutrient-rich waters), too. This latter observation suggests that temporal

and spatial lags between primary production and the availability of prey may not be as pronounced for Mediterranean sperm whales as they are in other parts of the globe, such as the tropical Pacific (Jaquet, 1996). Another study from the same researchers highlighted what appears to be a strong link between sperm whale distribution and fronts separating water masses in the deep pelagic waters of the north-western basin, especially the North Balearic Front to the north and west of the Balearic archipelago that separates the remnants of Atlantic surface water inflows from the colder waters of the Ligurian Basin to the north (Gannier and Praca, 2007). This relationship is not unusual for sperm whales, as studies from other regions have illustrated associations between sperm whales and sea surface temperature features such as warm-core eddies from the Gulf Stream in the North Atlantic (e.g. Griffin, 1999). These results again speak to an underlying similarity in the niches occupied by sperm whales in the Mediterranean to those of other populations.

The overall picture then, is that

- Mediterranean sperm whales live in the same way as neighbour from Atlantic Ocean, that is, where mesopelagic squid are.
- The limited accessibility to this ecological niche from human fisheries activity has probably been an advantage for Mediterranean sperm whales, considering that currently 85% of assessed fish stocks in our basin are harvested unsustainably (Colloca *et al.*, 2013).

Despite these insights, important gaps in our understanding remain. Our knowledge is strongly restricted to some months of the year, for example. Data are currently very limited in the winter months because of the more challenging weather conditions, but hopefully new passive acoustic technologies will improve this picture in the coming years. Surveys using moored devices have been able to confirm that sperm whale presence in the Ligurian Sea is not limited to summer months (fig. 1.2.1), with animals recorded in the northeast zone in December (Giorli *et al.*, 2016), although surveys using towed hydrophones on a wider spatial scale still suggest some seasonality in sperm whale presence (Laran and Drouot-Dulau, 2007). Giorli *et*

al. (2016) also reported an apparent shift towards concentrating foraging at night outside of the summer months in their study area, which could indicate seasonality in either prey behaviour or main prey species. Nonetheless, more information on seasonal patterns in habitat use should be an important ongoing research goal, not least because economically costly conservation actions are more robustly defensible when based on knowledge of where key habitat is found at different times of the year.

Figure 1.2.1: sperm whale in front of Bergeggi island - Vado canyon, Ligurian Sea (©Biagio Violi – Menkab, il respiro del mare)

From the technique of tracking isotope signatures across growth layer groups in the teeth of stranded animals, more conclusions have been obtained on the habitat use and movements of this predator within Mediterranean. Mendes *et al.* (2007) analysing teeth of stranded individuals from Iceland, Scotland, Azores and Greece get interesting results: animals from the Mediterranean had lower δ15N levels than other samples, because of the oligotrophic nature of the Mediterranean Sea - δ15N depletion is characteristic of reduced levels of nitrate assimilation by phytoplankton; δ15C levels were instead not unusual within the other analysed samples, indicating that the animals not use food sources that were closely associated with coastal waters, which is consistent with all the evidence above that Mediterranean sperm whales have a similar mainly squid-based diet to sperm whales all over the

world (Mendes et al . 2007), as also shown by the recent analysis of food preference of sperm whales in the Mediterranean (Foskolos *et al.*, 2020). This dependence on deep-sea squids is surely a key factor in the persistence of such a large predator in the oligotrophic Mediterranean Sea, since it confers a degree of independence from the main food chain that passes from phytoplankton to zooplankton to fish in the surface waters, although just how independent they can be remains to be seen.

1.3 Age/sexes classes

An interesting point that need to be deeply discussed within sperm whale social structure is the segregation between age and sex classes. In the oceans, sperm whales have strong divisions among age/sex classes (Whitehead, 2003; Whitehead and Weilgart, 2000; see fig. 1.3.1 from Whitehead, 2018) as here described:

- *tropical and subtropical social units* of females, calves and juveniles (fig. 1.3.2).
- *mid-latitude groups* of maturing males (called 'bachelor' schools).
- *high latitude* of singleton mature males.

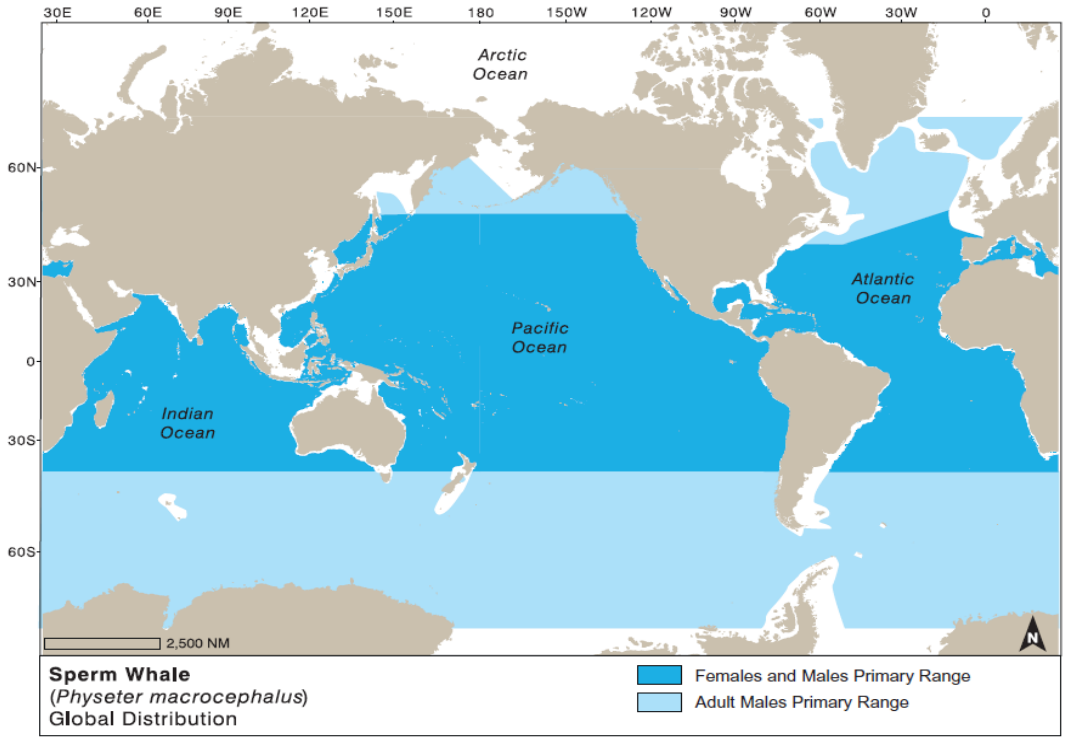


Figure 1.3.1: sperm whale age/sex distribution in the ocean.

Pictures from Whitehead, H. (2018). Sperm whale: Physeter macrocephalus. In Encyclopedia of marine mammals (pp. 919-925).

The Mediterranean Sea has a restricted latitudinal range and probably has not the conditions and features for such segregation (Rendell and Frantzis, 2016). Thus, the described classes probably inhabit the same areas, but they are not closely associated. Gannier *et al.* (2002) didn't detect any breeding groups in the north-western basin (above 41° latitude), where sperm whale relative

abundance was high. However, due to the relatively small size of the western Mediterranean Sea, breeding schools close to the Balearic Islands would be separated by less than 200 NM from other whales in the Gulf of Lions. It is tempting to think of the overall picture as a microcosm of the situation found in oceans, particularly the nearby Atlantic, where males and bachelor groups tend to feed in higher latitudes – during the summer - and breeding schools are more constrained to temperate/ sub-tropical latitudes (Rice 1989). In regions as the Hellenic Trench, social units are composed of about eight individuals on average and have a much more fluid structure in comparison to open ocean populations, because of some individuals that often change units. The most interesting point emerged in this area is that solitary males, loose male aggregations, social units and small bachelor groups seem to coexist all year around (Frantzis *et al.*, 2014). In details, Frantzis *et al.* (2014) classified the encounters as follow:

Solitary male: a single male with no other sperm whales detectable visually or acoustically for at least 2 h before and 2 h after the visual encounter.

Male aggregation: a loose aggregation of sub-adult or adult males spread in a radius of usually up to 10, but possibly up to 20 km (two out of 45 such cases encountered). Whales usually follow independent dive cycles without approaching each other to less than c. 2 km. Rarely, especially when young sub-adult males are present, whales may approach one another and synchronize their dive cycles and flukings, come into visual or even physical contact while socializing and/or producing social codas (sensu Frantzis and Alexiadou, 2008).

Social unit: stable group of 4 to 13 or more whales that either include calves among them, or have been encountered together more than once in different years; they may be encountered either as a close formation socializing at surface or travelling together, or dispersed at a radius of up to 20 km while foraging. Social units may be encountered with occasional male or female visitors among them.

Unclassified: an encounter that cannot be classified with certainty in any of the above encounter types because it ended before all the necessary data could be collected or represents small groups or individuals in a phase of transition between the above encounter types.

Similarly, on the other side of Italian peninsula, off the Bay of Naples all the major types of groupings that have been identified elsewhere (female social groups, singleton males and bachelor groups) have been observed too (Pace *et al.* 2014). Moving to the west, the same coexistence of age/sex classes has been found in the waters around the Balearic Islands (Pirotta *et al.*, 2011).

All these studies make emerge two important findings: **(1) a compressed social structure within Mediterranean Sea compared to their ocean counterparts**, and **(2)** a **possible intraspecific competition for prey resources among age classes** (Rendell and Frantzis, 2016).

Figure 1.3.2: sperm whale unit, in the waters of Pico – Azores (© Biagio Violi – Futurismo Azores Whale watching).

In conclusion, sperm whale age/sex classes which are found elsewhere in allopatry, in Mediterranean are in sympatry. We don't know whether this reflects a fundamentally different behavioural response or simply a latitudinal compression of the 'normal' pattern of age/ sex segregation found in the

oceans. Nonetheless, repeated observations in the Mediterranean reveal a sympatry of solitary males and female groups, with no obvious reproductive purpose (Frantzis *et al.*, 2014; Pace *et al.*, 2014a; Pirotta *et al.*, 2011), that is not typically seen in other areas (Whitehead, 2003). In the open ocean, although there is some evidence of seasonality in the density of mature males on the breeding ground (Whitehead, 2003), in some areas such as off the Galapagos Islands (Hope & Whitehead, 1991) and northern Chile (Whitehead, 2003), some large mature males seem to remain on the breeding grounds throughout the year. Off the Galapagos Islands, males have been shown to rove between groups of females, spending only a few hours with any one group (Whitehead, 1993). Re-association of males with one group often occurred over periods of a few days, but never over more than one week (Whitehead, 1993).

Because of the described overlapping ranges of different sex classes, the way that these classes of sperm whales divide up ecological niches may be altered and lead to an increasing competition for resources between female groups and solitary or very loosely associated juvenile males (Rendell and Frantzis, 2016). Understanding whether there is this competition because of sympatry, that is not seen in other oceans, is a key point: if lactating female sperm whales are facing competition for resources from subadult males that they do not face in other populations, this could lead to constraints on population growth rate that are not predicted by studies outside the Mediterranean Sea (Rendell and Frantzis, 2016).

1.4 Group size

It is well known that cetacean group sizes derive from a balance of evolutionary and ecological forces that can change in both space and time (Connor, 2000). As in the oceans, the widespread distribution of sperm whales in the Mediterranean Sea is likely to be linked to food resources. Some interesting results in sperm whale group size and distribution within the Mediterranean Sea come from Drouot *et al.* (2004), through data collected from 1997 to 2002 (fig 1.4.1). In that study, variations in the size of sperm whale schools/underwater aggregations were assessed using both visual and acoustic data.

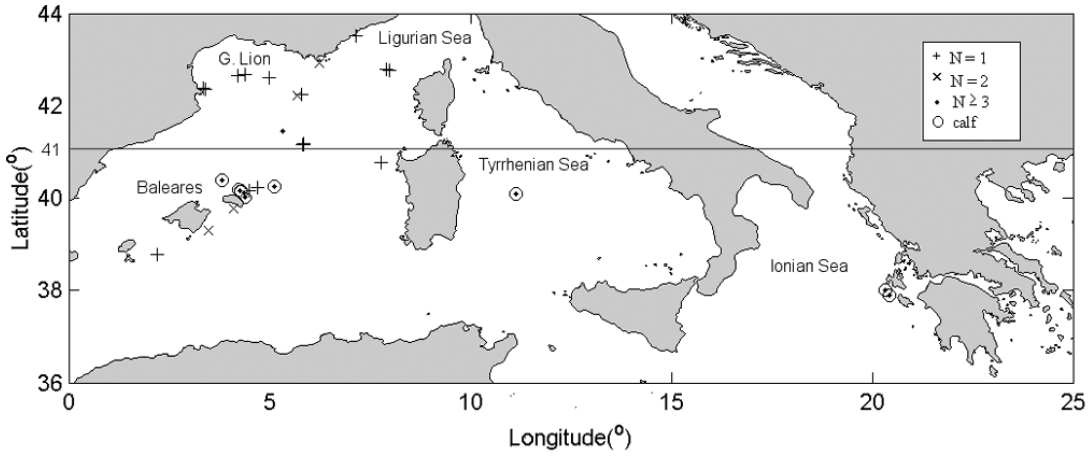


Figure 1.4.1: Map with group size distribution in the Mediterranean. (Pictures from Drouot V., Gannier A., Goold J.C. 2004. Summer social distribution of sperm whales (Physeter macrocephalus) in the Mediterranean Sea. J. of the mar. Biological Association of the United Kingdom 84(3): 675-680).

In this study, regional comparisons were undertaken, considering the 41° parallel as a north/south boundary: in the southern region, schools of up to seven sperm whales were sighted and calves were relatively frequent; in the northern region, school sizes were significantly smaller, with a maximum of three whales sighted at the surface. These summer surveys demonstrated a segregation of males, in the north, from larger schools including calves, which seemed to be confined to the southern region (Drouot *et al.*, 2004). In general, both surface sightings and underwater aggregations seem to increase significantly in size from the northern to the southern regions of the

Mediterranean Sea. However, some observations of social units have been done in the northern part of Western basin: Moulins and Würtz (2005) reported a 'herd' of 10 females and calves sighted in the Ligurian Sea off Monaco in 2001, Calogero *et al.* (2019) sighted a group of three individuals, two adults and 1 calf and filmed with a drone the breast feeding behaviour in Ligurian Sea (fig. 1.4.2).

Figure 1.4.2: mum and calf during lactation in Ligurian Sea
(© Gabriel Principato – Artescienza / Menkab, il respiro del mare)

These three cases seem to be a rare and/or exceptional observation for northern part of the western basin – despite all occurred in autumn/winter… - but need to keep in mind in the challenge of understanding group size and social structure in the Mediterranean. Moving to the Gulf of Lions, aggregation of sperm whales might result from the increased productivity of the area, characterized by numerous submarine canyons and frequent upwelling events (Gannier *et al.*, 2002). Along the Hellenic Trench, Frantzis *et al.* (2014) in their surveys from 1998 to 2009 have identified 16 social units, with 13 resighted across multiple years, containing 4 to 13 individuals, with an average of eight. In the same study, several instances of apparently temporary aggregations containing multiple social units with up to 15 individuals present were also observed, and on four occasions, 'gatherings' containing 17 to 20 individuals

were sighted within a 10 km radius, comprising either two social units or a social unit with a loose aggregation of males.

It is possible to speculate that female social units in the Mediterranean may be smaller than those encountered in the Pacific and the North Atlantic, which typically contain around 11 to 12 females and immatures (Whitehead *et al.*, 2012). However, in the Gulf of Mexico and Dominica, two populations have shown comparable unit sizes, and social units contain five to six individuals on average (Whitehead *et al.*, 2012). It is possible then that similar evolutionary and ecological forces are acting on social unit size both in these latter populations and in the Mediterranean, although the nature of these forces remains a matter of speculation (Rendell & Frantzis, 2016). It has been suggested that sperm whales changed the group size as a response to variations in the distribution and abundance of their food. Recent work from Jaquet & Gendron (2002), based on squid catches in the Gulf of California, tends to confirm that the size of sperm whale aggregations is related to the size of prey patches. However, sightings of 10 to 30 individuals were reported in the Mediterranean Sea in the past (Bolognari, 1951; Mangano, 1983), suggesting that the basin may have/had the potential to sustain larger sperm whale schools. A decrease in sperm whale school size could result from the impact of human activity, as will be discussed in paragraph 1.9-1.10 (Rendell and Frantzis, 2016).

1.5 Body size

Variation in ecological conditions can result in varying growth patterns in different populations of the same species. In the oceans, while adult females reach about 11 m in length and 15 t, a physically mature male is approximately 16 m and 45 t (Rice, 1989) (fig. 1.5.1).

Figure 1.5.1: adult male fluking with calf head on its side - Pico, Azores (©Biagio Violi – Futurismo Azores Whale watching)

As well known, sperm whales perform deep dives in search of food, during which they emit an almost continuous sequence of loud impulsive clicks for echolocation (Mohl *et al.*, 2000). Sperm whale clicks are made up of several regularly spaced pulses resulting from multiple reflection of the initial sound within the head of the animal. The spacing between the pulses within a click, termed inter-pulse interval (IPI), has been demonstrated to be related to the size of the animal (Gordon, 1991). Analysing the distribution of IPI values within several regions in Mediterranean, results indicated that the IPIs were consistently greater in the northern basin than in the southern areas. Body length extrapolations indicated that the whales detected in the north were principally large animals, around 12 m long, thus probably sexually mature males (Drouot *et al.* 2004). These results were consistent with those of Pavan

et al. (1997), which gave mainly length estimates of 11 m to 12 m for whales detected in the northern basin, although they mentioned that large males (13 to 14 m long) were also detected in the southern regions of the Mediterranean (south Tyrrhenian Sea and Ionian Sea). The three-year seasonal study of Marini *et al.* (1996) in the central Tyrrhenian Sea showed that the maximum whale size encountered was 13.5 m. In the southern regions, the individual size estimations from IPIs indicated a more heterogeneous population, including young and adult whales (Drouot *et al.*, 2004). Along the Hellenic Trench, similar acoustic measurements suggested a mean length of 11.4 m and a range of 8.9–14.6 m for 19 males, and a mean of 9.1 m with a range of 8.6–9.5 m for 9 females (Frantzis and Alexiadou, 2008, Frantzis *et al.*, 2014). All results obtained in Mediterranean have the same trend as a study carried out in the northern Atlantic (Adler-Fenchel, 1980), which showed that sequences recorded at lower latitudes, where females and immature males are found, had shorter IPIs than those from higher latitudes where only large males are found.

The occurrence of whales between 15 and 19 m have been reported until 1995 along the Italian coast and, from 1986 to 1998, out of the 27 sperm whales reported entangled or stranded (with evidence of drift-net fishing interactions such as piece of net around the fluke), 77.8% were greater than 12m in length (Centro Studi Cetacei, 1986-1998). From strandings analyses, there have been no reliable records of stranded sperm whales that exceeded 15 m in total length (Rendell and Frantzis 2016). Here is a list of main length records:

- Frantzis *et al.* (2003) count growth layer in tooth sections from two stranded whales, revealing a female of 25 years old and 10 m long, plus a male 44 years old and 12.8 m long.
- Bearzi *et al.*'s (2011) exhaustive survey of strandings in the Adriatic Sea revealed only one instance of a whale greater than 15 m in length.
- Mazzariol *et al.*, (2011), from a recent mass stranding in the Adriatic consisted entirely of males, all measurements were less than 12.5 m in length.

- Mazzariol *et al.* (2018), from a second mass stranding of 4 individuals (3 females and a foetus) in Adriatic coast, recorded measurements no longer than 8.95 m.
- Fosklos *et al.* (2020), in analysing stomach content of 10 stranded sperm whales along Hellenic Trenches, recorded body length of 7.9 m for females and 10.5 m for the males.

In the framework of body size analyses, it is hard to say whether this size difference is due to an evolved reduction in growth in this population or a phenotypic response resulting from a lack of access to the highly productive high latitude waters in which male sperm whales outside the Mediterranean Sea grow to their mature lengths (Whitehead and Weilgart, 2000), but it does suggest that Mediterranean sperm whales may be subject to different ecological and evolutionary forces than those experienced by their open ocean counterparts (Rendell and Frantzis, 2016).

1.6 Diving and feeding behaviour

Sperm whale is one of "champions" in freediving "competition" among marine mammals. A typical sperm whale dive cycle consists of a 40-50 min dive followed by a surface period of about 8 min for breathing (Gordon, 1987; Papastavrou *et al.*, 1989; Whitehead *et al.*, 1992). The diving period can be divided into three phases: (1) a descent phase (preceded by the fluke-up, where the whale descends almost vertically from the surface to the foraging depth), (2) a foraging phase (where the whale does a horizontal movement at the foraging depth in search for food), and (3) an ascent phase (where the whale returns to the surface) (Gordon, 1987; Watkins *et al.*, 2002; Zimmer *et al.*, 2003; Watwood *et al.* 2006). Because sperm whales feed at great depths, they have never been directly observed while feeding and, therefore, their feeding methods can only be inferred. Indeed, several authors (Goold & Jones, 1995; Gordon, 1987; Norris & Harvey, 1972; Weilgart, 1990) suggest that sperm whales use echolocation to detect prey at ranges of several hundred meters. During the descent and foraging phases of the dive they produce "regular clicks" almost continuously and emitted with regular rate, between 0.5 and 2 clicks per s (Drouot, 2003; Goold & Jones, 1995; Gordon, 1995; Weilgart & Whitehead, 1988). Sperm whale clicks are made up of a number of regularly spaced sound pulses resulting from multiple reflection of the initial sound within the head of the animal (see fig. 1.6.1). The time spacing between pulses in a click, termed inter-pulse interval (IPI), has been demonstrated to be a function of the body length (Goold & Jones, 1995; Gordon, 1991) – as discussed in the previous paragraph. The long sequences of regular clicks are spaced with "creaks," defined as an increased click rate of up to 220 per s, persisting for between 10 and 25 s, and followed by a silence (Gordon, 1987). Creaks are thought to be produced by sperm whales investigating targets at close range and, therefore, to be indicative of feeding attempts (Goold, 1999; Gordon, 1995; Mullins *et al.*, 1988). The increasing click rate during a creak may reflect decreasing distance to the target. Leaper *et al.* (1992) assumed sperm whales

emit regular clicks up to 50% of the time, in agreement with the proportion of 48% found by Whitehead & Weilgart (1990) in the Galapagos.

Figure 1.6.1: sperm whale skull - Natural History Museum of Edinburgh. (©Biagio Violi – Menkab, il respiro del mare)

However, these average clicking rates were obtained for breeding groups; mature males, as found by Gordon & Steiner (1992), around the Azores can be acoustically active for 75% of the time when engaged in cycles of long feeding dives. Mature males feeding off New Zealand were shown to spend approximately 72% of their time clicking when engaged in cycles of long feeding dives (Gordon *et al.*, 1987; 1992). If a creak signifies a feeding event, and that each event represents the successful capture of at least one squid, the average of 25 creaks produced per dive would correspond to at least 25 feeding attempt per dive cycle. Therefore, with an average dive cycle of 55 min, and considering whales are involved in diving activity about 80% of the time, it could be extrapolated that around 750 squid are being eaten per day (24-h period). If there are multiple prey captures at each feeding event, then this figure would increase accordingly - Note that whales would creak into shoals of squid rather than chasing individual prey - (Gordon & Steiner, 1992). There is a conjecture that some squid may be detected visually, without the use of echolocation (Fristrup & Harbison, 2002), and some cephalopod families such

as Histioteuthidae and Ommastrephidae are known to have bioluminescent organs and could be conspicuous at depth (Clarke, 1985). If there is some visually targeted feeding, based on bioluminescence, such feeding events could explain the short but periodic interruptions of the regular click sequences which often occur (i.e., short periods of silence between long sequences of clicks); however, even without visually based foraging, Clarke's (1987) prey capture calculations, from studies of stomach contents, suggested that the number of cephalopods eaten ranges from around 800 to 2,000 over periods of 1 to 2.5 days (Clarke, 1987).

Within Mediterranean Sea, sperm whales exhibited dive cycle dynamic as showed in other parts of the world, about 45 min diving, 9 min surface period, with 5 blows/min and a horizontal displacement of 1.3 nm between dives. As elsewhere, sperm whales produce echolocation for 70% of the time clicks during an entire dive cycle (i.e., including the surface period) (Drouot *et al.*, 2004). Recently, D-Tag methodology gave interesting results about diving behaviour of sperm whales in the Ligurian Sea: after fluking, sperm whales start clicking at 96.7 m depth and foraging at 635.6 m depth (see fig 1.6.2 from Watwood *et al.*, 2006). Sperm whale click rates increase rapidly to produce a 'buzz' while it is attempting to catch a prey, and these buzzes are associated with rapid changes in direction (Miller *et al.*, 2004). Dtag suction cup studies, performed in Ligurian Sea by Teloni (2005) found that whales in the Ligurian Sea produced in average 18.5 numbers of buzzes per foraging dive. Using surface hydrophones rather than on-animal tags, Gannier *et al.* (2012) measured an average of 25 buzzes per dive from a larger sample of 156 dives during 52 sperm whale sightings. Drouot et al (2004) showed the first creak of the dive occurred consistently around 6 to 7 min after the whales fluked-up. This would imply, considering a descending speed of between 75 and 120 m/min (Drouot, 2003; Gordon, 1987; Lockyer, 1977; Madsen *et al.*, 2002; Mullins *et al.*, 1988; Papastavrou *et al.*, 1989; Watkins *et al.*, 2002), a foraging depth of around 490 to 780 m. This implication is consistent with recent work from time-depth recording tags showing that the first creak of the dive is emitted as the whale reaches the foraging depth where it levels off (Zimmer *et*

al., 2003). Potentially, these results confirm that sperm whale foraging behaviour in the Mediterranean appears to be very typical of the species and strongly support the hypothesis that this population occupies a very similar ecological niche to those in the oceans.

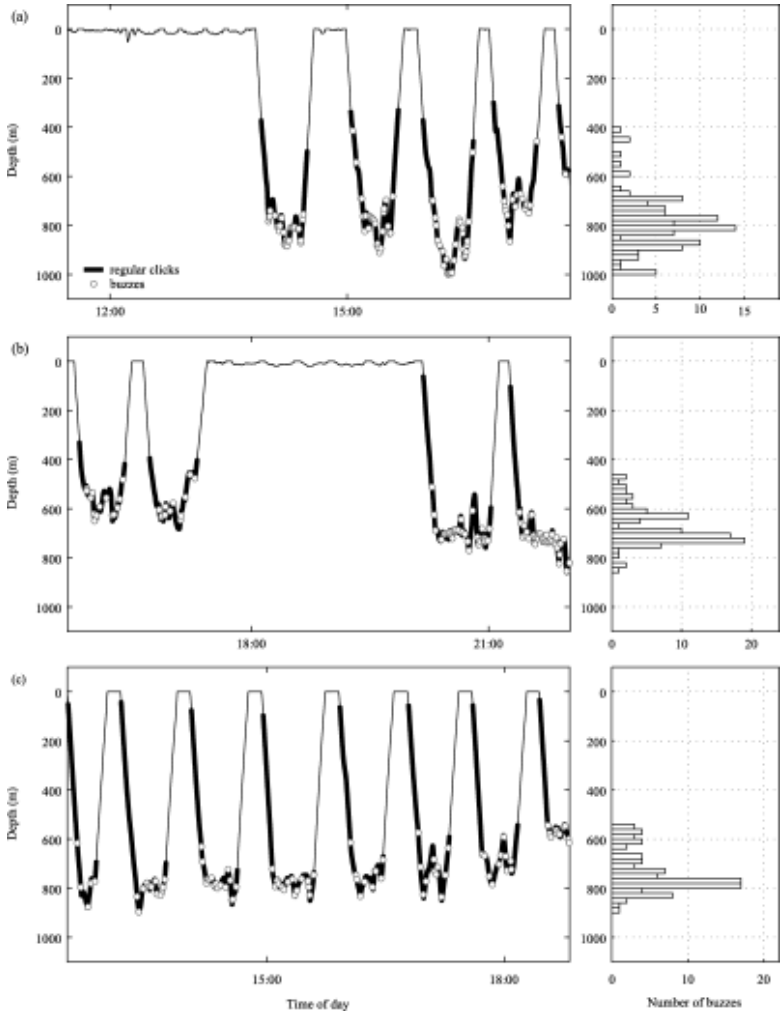


Figure 1.6.2: Dive track and histogram of depth of buzz production. Recorded in whales from (a) the Atlantic Ocean, (b) the Gulf of Mexico and (c) the Ligurian Sea (Picture from Watwood SL, Miller PJ, Johnson M, Madsen PT, Tyack PL. 2006. Deepdiving foraging behaviour of sperm whales (Physeter macrocephalus). J Anim Ecol. 75(3):814–825)

The described diving behaviour is consistent with the presence in the midwater habitat of Mediterranean of squid species such as *Histioteuthis bonnellii* and *Histioteuthis reversa* (Mangold & Boletzky, 1987; Roberts, 2003, Foskolos *et al.* 2020), known to be part – and probably the main one - of the sperm whale diet (see next paragraph for more details). Interestingly, again

Drouot *et al.* (2004) observed the same time interval between "the fluke-up to the first creak of the dive" and "the last click of the dive to the surfacing moment" (i.e., 6.6 min). Assuming the whale stops clicking as it starts its vertical ascent to the surface and considering an ascending speed of about 117 m/min, these results suggest that sperm whales remain in the same depth layer during the dive to chase prey. The whales observed during this study measured between 11.8 and 13.8 m long and were probably all males (Rice, 1989). The results showed that the number of creaks produced during the dive and the timing of the first creak increased significantly with whale size. This is coherent with the biological needs of the animal: the larger the whale is, the more food intake it requires. The increasing time between the whale fluke-up and the first creak of the dive might reflect an increased travel time to reach the foraging layer, suggesting that larger individuals tend to reach deeper layers to search for food, perhaps to find larger prey items or a higher abundance of squid. Parallelly, this trend could also reflect a lesser diving capacity in smaller whales (Drouot *et al.*, 2004).

1.7 Diet and preys

The sperm whale is a key top predator of the meso and bathypelagic zones in the oceans of the oceans (Whitehead, 2003). The actual knowledge we have is based on examination of stomach contents from stranded animals, assessment on stable isotopes profiles, and analysing echolocations behaviour in relation of habitat use. Several scientists assessed sperm whale diets thanks to stomach content analyses from dead animals, catch by whaling activity or found stranded. Many studies of the diet of the sperm whale have shown that, except off Iceland (Clarke & MacLeod 1976; Roe 1969; Martin & Clarke 1986) the main food of the whale comprises cephalopods (see Clarke 1980, 1986; Garibaldi and Podestà, 2014; Fosklos *et al.*, 2020). Because the males can exceed 60 t and females can exceed 30 t, they are a major predator of cephalopods (Clarke 1985) and probably consume annually a considerably greater mass of cephalopods than the total mass of all fish caught by man. In a single whale's stomach, the chitinous beaks are not digested and can be over 18000. The specific identification of such collections has greatly extended our knowledge of cephalopod predators' food and migration habits (Clarke 1986). Clarke *et al.* (1993), analysed the stomach contents from 17 sperm whales (15 males and 2 females) haunted for commercial activities in 1981-1984 in the Azores archipelago, one of the most important breeding and feeding area in Atlantic Ocean (fig. 1.7.1). In total 28738 cephalopods and 16 fish were identified and measured. Furthermore, tunicates in two whales and man-made products in three whales have been found. None of the stomachs was empty. In percentage, the stomach content was represented for 94.1% by flesh and 5.9% by indigestible fragments alone, that is, beaks of cephalopods. Respectively, 12 species of fishes and 40 species of cephalopods have been identified by the flesh and lower beaks analyses. Eight cephalopod families have been confirmed to be the main meal for this species in this region: the Octopoteuthidae (39.8%), the Histioteuthidae (32.7%), the Architeuthidae (12.1%), the Lepidoteuthidae (4.5%), the Ommastrephidae (3.4%), the Pholidoteuthidae (2.1%), the Cycloteuthidae (1.9%), the Cranchiidae (1.7%) and

eight other families each contributing less than 1 % by mass. An interesting finding has been the presence of *Gonatus* beaks, and other cephalopods, not previously recorded from the North Atlantic, such as *Onychoteuthis borealijaponicus*, *Histioteuthis bonnellii corpuscular* and *Histioteuthis miranda* as a proof that some whales have migrated southwards to the Azores.

Figure 1.7.1: sperm whale feeding in the southern water of Pico – Azores (© Biagio Violi – Futurismo Azores Whale Watching)

The presence of a large Megalocranchia species, proof of migration from higher latitudes off Iceland. The presence of *Teuthowenia maculata* shows which whales came north from the West coast of Africa. Species not recorded previously in the diet of sperm whales in the North Atlantic were *Ommastrephes bartrami, Gonatus steenstrupi, H. meleagroteuthis, Discoteuthis laciniosa, Mastigoteulhis* species, *Chiroteulhis* species, *Helicocranchia, Liocranchia reinhardti,* and *PLiguriella.* Another interesting finding was that: 77.5% of the species eaten have luminous organs and 82% of the species have neutrally buoyancy. Thus, it seems likely that the sperm whale food is represented about 80% by slow-swimming and neutrally buoyant squids and the left 20% is represented by faster swimming and larger cephalopods (Clarke *et al.*, 1993).

Within the Mediterranean "menu", as shown from stomach contents of several odontocete species (Orsi Relini & Garibaldi 1992, Würtz *et al.* 1992, Würtz & Marrale 1993), Histioteuthidae family represent the main meal for deep divers that rarely feed on fishes and octopus. In detail, within the Western basin, stomach contents have been assessed by:

- De Stephanis *et al.* (2013), who examined stomach contents from a stranded animal along Spanish shoreline and found some squid beaks in the abdominal cavity and on the exterior portion of the small intestine, but a large mass of compacted plastics have been seen protruding through a rupture in the first stomach compartment.
- Garibaldi and Podestà (2014) from a male stranded in the west coast, found 233 upper and 291 lower beaks, more or less in the same digestion rate, 288 of them belonged to *H. bonnellii*, 2 to *Galiteuthis armata* and 1 to *Octopoteuthis sp.*, and considering that no flesh were found, authors assumed that the whale didn't feed in the last days.

In the eastern basin, four interesting assessment have been done.

- Roberts (2003) analysed the stomach of a male sperm whale found floating dead near Crete (Greece), contained nearly 3000 squid beaks from seven squid species, mainly *H. bonnellii,* followed by *O. sicula*, *H. reversa, Ancistroteuthis lichtensteini, Chiroteuthis veranyi veranyi, Onychoteuthis banksia, Ancistrocheirus lesueuri.*
- Mazzariol *et al.* (2011), examined the stomach content from 7 stranded sperm whale in Adriatic Sea in 2009, found members of the Histioteuthidae family were the commonest prey, specifically *H. bonnellii* and, to a lesser extent, *H. reversa* and other rare species (*Octopoteuthis sicula* and *Galiteuthis armata*).
- From a second mass stranding event in Adriatic Sea in 2014, Mazzariol *et al.* (2018) found eight cephalopod species. The 7,539 recognizable lower beaks, were classified in the family Histioteuthidae and correspond for the 71% to *H. bonnellii* and 24% to *H. reversa*, followed by *A. lesueurii* and *O. sicula*. All these cephalopods are meso or bentho pelagic species, inhabiting deep waters and are not present in the Central and Northern

part of the Adriatic; it is therefore evident that they were preyed upon in the Ionian Sea or in the Southern Adriatic.

- To date the important dataset came from the analyses of the stomach contents examined by Foskolos *et al.* (2020) of nine individuals stranded in Greece between 2005 and 2014. Other than the expected *H. bonnellii* and *H. reversa* the and octopus squid *O. sicula*, found ten prey species not previously reported for sperm whales in the Mediterranean Sea, respectively nine cephalopods and one teleost: *Brachioteuthis riisei* (Steenstrup, 1882), *Chtenopteryx sicula* (Véerany, 1851), *Abralia veranyi, Abraliopsis morisii* (Véerany,1851), *Ommastrephes sp., T. sagittatus, Pyroteuthis margaritifera* (Rüppell, 1844), *Heteroteuthis dispar*, *Octopus vulgaris* (Cuvier, 1797) and the fish *Chauliodus sloani*. This high diversity among sperm whale preys is due to the greater number of stomachs that have been analysed comparing to previous assessments. Except for *Ommastrephes sp.* and *T. sagittatus*, the listed species are small-bodied preys. In the same study, the presence of *Histioteuthis* species among non-calves was observed to range from 2648 kg to 4100 kg.

In the Mediterranean Sea, squids are not as big as in the oceans. The only species highly energetic that can be found are ommastrephid squids, that is, *Todarodes sagittatus* (Lamarck, 1798), *Ommastrephes bartramii* (Lesueur, 1821) and *Thysanoteuthis rhombus* (Troschel, 1857). However, they are not within favourite preys of sperm whale, but all listed studies confirm that *H. bonnellii* is the main one. Garibaldi & Podestà (2005) suggested that sperm whales catch this species that undergoes significant vertical migrations, even if the largest specimens are preferably found in deeper regions. As confirmed by Voss *et al.* (1992), larger juveniles and sub-adults have been found at night in the Eastern Atlantic between 200 m and 800 m.

Lockyer (1981) estimated the **daily feeding rate** of sperm whales consuming cephalopods at 3% of the body weight, though this may vary, possibly up to 4-5%, depending on prey items (Clarke *et al.*, 1988). If this estimate is accurate it is expected that an individual normally consumes 660 to 1030 kg per day and from 240 to 377 tn per year. Feeding on these preys, sperm

whales presence is linked to the deep sea regions and therefore (partially) independent from the food chain that passes through surface waters: all of this justify the presence of such predators in an oligotrophic sea as Mediterranean (Rendell and Frantzis, 2016).

1.8 Acoustic review

One of the most fascinating features of sperm whale is the production of a highly directional and extremely powerful pulses of sound (Møhl *et al.*, 2000; Zimmer *et al.*, 2005), that is the most intense sonar among living animals, and is used to sense the surrounding deep ocean environment, detect and pursue prey (Miller *et al.*, 2004). In detail, sperm whales are known to emit different types of vocalizations which are made of series of clicks (Whitehead & Weilgart, 1990). These vocalizations have been classified in regular clicks, slow clicks, chirrups and codas. These sounds patterns differ mainly by the rate at which clicks are emitted. As explained in the previous two paragraphs, regular clicks are emitted during foraging dives, are directional and characterized by an intense and forward-directed beam (Møhl *et al.* 2000; Madsen *et al.* 2002). Source levels within the beam are estimated to be as high as 236 dB re 1 lPa at 1 m. (Møhl *et al.* 2003). Several acoustic studies proved that echoes from both the surface and the sea bottom are detected by the tags attached on the head of a whale, and suggest that the whale may use these echoes for orientation and navigation (Johnson & Tyack 2003; Zimmer *et al.* 2003). On the other way, codas are series of 3 to 20 clicks matching a distinctive and repetitive pattern (Watkins & Schevill, 1977). Codas have been identified as social vocalizations and are believed to play a major role in acoustic communication, although their function is not well understood. While socializing, whales emit extensive coda repertoires generally when at the surface (Whitehead & Weilgart, 1991; Gordon, 1987; Watkins *et al.*, 1985), although codas have also been recorded during prolonged dives. Populations can be divided in sympatry by these dialects (Rendell and Whitehead, 2003), and some types of codas can also identify subpopulations (Gero *et al.*, 2016).

In the Mediterranean Sea, initially it was suggested that a single coda pattern exists (Borsani & Pavan, 1994; Pavan *et al*, 1996). This coda is composed of 4 clicks and is described as a "3 + 1" coda, because the last click is emitted with a wider inter-click interval compared to the first three ones. For a long time, this coda pattern was believed to be the unique coda of the

Mediterranean, although other codas were reported on few occasions (Pavan *et al.* 2000; Borsani & Pavan, 1994). Drouot et al (2004) within Mediterranean Sea, classified coda on the base of the measurement of Inter-Click Intervals (ICI), as here is described (fig. 1.8.1): *Regular* codas: all clicks evenly spaced (all ICI equal); "3 +" codas: 3 first clicks evenly spaced ($ICI_1 = ICI_2$), forming a root, and the following clicks have greater ICI than the average ICI of the root (($ICI_1 + ICI_2$)/ 2); "3 ++" codas: similar to "3 +" codas, but with the click following the root having ICI more than 3 times the length of the average ICI of the 3-click root; *Undefined* codas: codas not falling into any of the three categories described above.

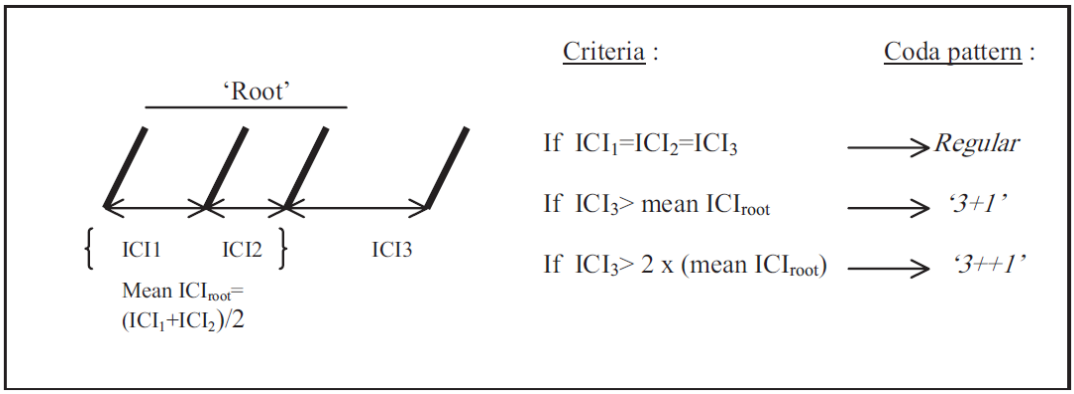


Figure 1.8.1: Coda classification (from Drouot et al., 2004)

As in the oceans, sperm whales of Mediterranean make codas while socialising, but also at the beginning and at the end of dives. Frantzis and Alexiadou (2008) studied codas produced only by male sperm whales along the Hellenic Trench. They analyzed 615 codas and described 8 coda families and 25 distinctive coda types. Among them they described codas of a type termed 'root', with shorter duration compared with other types. 'Root' codas had a series of very rapid clicks at the start, produced at the surface, and associated when regular foraging dive cycles had been interrupted probably by the presence of disturbances, such as the proximity of the research vessel, or a swimmer entering the water. Thus, Frantzis and Alexiadou (2008) suggested that these root-type codas might function as alarm calls. Pavan *et al.* (2000)

found that 134 analysed codas (97% of the dataset) from 15 encounters spread in 12-year, recorded in the Ligurian and Tyrrhenian Seas were of the 3 + 1 pattern. Again, Teloni (2005) found that 128 analysed codas (98% of the dataset) from 27 sightings in the years 2001 to 2003, recoded in the wester basin had the same pattern. However, the codas analysed by Pavan *et al.* (2000) and Teloni (2005) come from singletons, presumably males. A wider acoustic analysis has been performed by Drouot *et al.* (2004): 751 codas recorded from 13 different encounters from Western and eastern Basin and including social units. Also, in this analysis, the pattern 3+1 was found in 67% of all the codas recorded. But the regularly spaced five-click coda was found as well. This latter is common to different areas of the world ocean, such as the West Indies (Moore *et al.*, 1993), where it represents a dominant pattern, the Galapagos (Weilgart & Whitehead, 1993), but also in the north western Atlantic (Watkins & Schevill, 1977). Differentiation in the Mediterranean dialect is reflected in the restricted exchange between groups of females and their offspring living in the Mediterranean Sea and those of the adjacent ocean.

In conclusion, the hypothesis of a single coda pattern present in Mediterranean would be very unusual compared to recordings made in other regions that report a much higher coda diversity (Rendell and Whitehead, 2003; Gero *et al.*, 2016), and soon after its formulation it was shown to be false. It has been confirmed that the acquiring of coda repertoires in a population is a phenomenon driven by cultural transmission (Rendell *et al.*, 2012). The presence within our basin of a coda that does not seem to be present in other regions is another indication of isolation for this population.

1.9 Stranding events

Sperm whale mass strandings are mysterious events. Threats as ship strikes (Pesante *et al.* 2001) entanglements in illegal fishing nets (Pace *et al.* 2008, Cornax & Pardo 2009) and ingestion of plastic debris (Mazzariol *et al.*, 2011) have been proposed as main causes. All these threats cause high mortality levels in this predators and dead animals can been found floating or stranded. Along eastern Italian coasts, the decline in the frequency of stranding events has been assessed by Bearzi *et al.* (2011) contrary to an expectation of increased reporting efficiency in modern times. Whitehead (2003) noted that whale's mass strandings have a clear strong social component, in which healthy animals that follow sick or confused ones move to a beach "doing as the group does". In the Adriatic Sea, sperm whale mass strandings have occurred five times since historical times, with the oldest known instance dating back to 1584. The occurrence of mortality events along this shorelines, where there is no suitable habitat for sperm whales, could lead to the idea that some regions in the upper Adriatic Sea are sort of 'sperm whale traps' (see Smeenk 1997, Goold *et al.* 2002, Pierce *et al.* 2007), where animals may become cornered for an extended period of time in areas containing not enough preys to sustain their daily request. In this area, at least 29% of live strandings (6 of 21) involved more than one individual. In addition, single individuals stranded dead or alive have been characterized by the presence of one or more other sperm whales at sea close by the stranding locations and sometimes stayed there for several days. Groups of sperm whales stranded in the Adriatic Sea were composed of 3-8 individuals and therefore small compared to the mean size of groups stranded outside of the Mediterranean Sea (e.g. Rice *et al.* 1986, Rice 1989, Christensen 1990), where stranding events occasionally involve 100+ animals (Evans *et al.* 2002). According to Brusina (1889) the majority of sperm whale mortality events occurred mostly along the central western Adriatic coast and were absent further south along the Italian coast (Brusina 1889). His idea was that sperm whales entering the Adriatic Sea moved north along the eastern coast, where waters are deeper, following the

main northbound circulation. At this point, he then speculated that when sperm whales wanted to go back, they followed the southbound stream along the Italian coast, where they often stranded in shallow waters. Impairment of the navigation and echo-location systems due to bathymetric features has been proposed also as a cause of "getting lost" in this area (Vanselaw *et al.* 2005).

Figure 1.9.1: stranded female in North Sardinia (@Luca Bittau – Seame Sardinia Onlus).

In December 2009, a pod of seven sperm whales stranded along the coastline of the Gargano Promontory (Italy), in the Southern Adriatic Sea. Three animals were still alive and died within 48 hours after stranding. The seven male sperm whales were 10.5 to 12.2 m long, with an estimated age between 15 and 25 years. In concordance with Brusina's theory, the cetaceans swam to the north and didn't find enough food and died because of starvation. This latter provoked the reduction of adipose body reserves and the consequent release into the bloodstream of chemical substances likely displaying neurotoxic and immunotoxic effects, altered the orientation and space perception of the whales, worsening their welfare and health (Mazzariol *et al.* 2011).

Figure 1.9.2: stomach content from stranded sperm whale in Greece. From Alexiadou et al. (2019) – (©Pelagos Cetacean Research Institute)

A second recent mass stranding event in Adriatic happened in 2014, when a pod of 7 animals stranded alive along the Italian coast of the Central

Adriatic Sea: 3 individuals died on the beach after a few hours due to internal damages induced by prolonged recumbency; the remaining 4 whales were refloated after great efforts.

All the dead animals were infected by dolphin morbillivirus (DMV). Their stomach content analyses revealed no recent feeding activities, and one had 1340 g of plastic bags, 1 jute bag and a piece of rope (Mazzariol *et al.*, 2018).

Several further strandings events happened in the last decade and the analyses of stomach contents reveal the presence of plastic debris, fishing nets and cords (as seen in de Stephanis *et al.*, 2013; Alexiadou *et al.*, 2019, see fig 1.9.2). Lastly in April 2019, a dead female sperm whale was found stranded in the North Est side of Sardinia (fig. 1.9.1). Again, in this latter event, some plastic has been found in the stomach (see fig. 1.9.3).

Figure 1.9.3: stomach content of stranded sperm whale in Sardinia (@Luca Bittau – Seame Sardinia Onlus).

1.10 Threats in Mediterranean Sea

The Mediterranean sperm whale population is a fragile entity. Its habitat, geographically restricted, can be considered as an 'oceanic island', hardly impacted by human activity, apparently cut-off from the much larger pool of conspecifics in the neighbouring Atlantic Ocean, and for these reason, the population is threatened (Rendell and Frantzis, 2016). Notarbartolo di Sciara (2014) identified six important human pressures faced by this predator: fishery bycatch (or 'ghost-fishing' by abandoned drift nets), ship strikes, ingestion of marine debris (above all plastics – as described in the previous paragraph), as main direct causes of death, but also some indirect ones such as chemical pollutants, anthropogenic noise and disturbance from poorly managed whale watching operations.

Figure 1.10.1: Propeller marks on stranded (A) and free ranging (B) sperm whales along the Hellenic Trench.
(Pictures from Frantzis, A., Leaper, R., Alexiadou, P., Prospathopoulos, A., & Lekkas, D. (2019). Shipping routes through core habitat of endangered sperm whales along the Hellenic Trench*, Greece: Can we reduce collision risks? PloS one, 14(2)).*

Large cetaceans in the Mediterranean Sea are indeed particularly susceptible to being hit by ships due to the high density of shipping routes over sensitive deep-sea ecosystems. Although this sea corresponds to only 0.8% of the world's oceans, carries about 30% of the world's total merchant shipping and 20% of its oil shipping. The total number of large cargo vessels that are cruising the Mediterranean Sea at any moment is >2000. Propeller marks and/or cut flukes have been observed on the body of different cetaceans' species (see fig. 1.10.1 from Frantzis *et al.*, 2019).

Entanglement in nets is the second major threat, and by far the most devastating impact has been from driftnets, called 'walls of death', averaging 20 km in length (Notarbartolo di Sciara, 1990). 229 stranded sperm whales that occurred in the north-western Mediterranean between 1971 and 2003 showed entanglement with nets and in many cases the nets were still present.

1.11 Whaling in Mediterranean Sea

Worldwide commercial whaling started in 1712 in North west Atlantic Ocean and moved in Pacific Ocean 85 yrs. later (Whitehead, 2002). Data on catches are available from 1800 to 1999 (Best 1983, Zemsky *et al.* 1995), but a gap exists between the beginning and when hunting move into the other oceans. Whitehead (2002) estimated that open-boat whaling reduced the worldwide sperm whale population to about 71% of its original level, and modern operations would have decreased it down to 32%. Within the Mediterranean Sea, there was no significant whaling activity, which was probably a crucial factor for the survival of the population of this basin. Despite, whalers knew and exploited the Gibraltar Straits ground, their efforts were focused mostly on the Atlantic side of the Strait. From 317 logbooks emerged only two expeditions into the Mediterranean itself, which led to a minimum removal estimate of 237 animals in the period from 1862 to 1899 (Aguilar and Borrell, 2007). It has also been reported that sperm whales were hunted more recently with explosives around the Straits of Messina in the years immediately following World War II (e.g. Bolognari, 1949), but accurate records were not kept so we do not know how many animals were killed during that time (Frantzis *et al.*, 2011).

1.12 Use of molecular ecology

Molecular analysis for population structure began in the 1960s with gel electrophoretic separation of allozymes (Lewontin & Hubby, 1966). These methods were widely used for several years until the discovery of the Polymerase Chain Reaction (PCR) by Kary Mullis in 1983, which allowed for the amplification of a specific region of DNA. This reaction was probably the most important one that revolutionized the development of DNA analyses. The next key step was the DNA sequencing techniques, which enabled us to read DNA sequences. Several types of molecular marker have been developed and used to address a variety of questions on population structure, evolutionary history, population dynamics and behaviour (Hoelzel *et al.* 2002). Lastly, next generation sequencing technology has provided ability to address evolutionary and ecological questions (Mardis, 2008). The use of markers with high levels of variation allows a better differentiation at the species, population and individual level. Some advances in molecular genetic techniques have resulted in the recognition of many new species in cryptic taxonomies (Mace, 2004). Although it is quite hard to define a cryptic taxon/species, Bickford *et al.* (2007) defined it as two or more species that are superficially indistinguishable each other for their morphology. Such cryptic taxa are of concern if they exist within taxa already classified as endangered: indeed, the risk of extinction is often greater in these cryptic taxa because of reductions in distribution and population size. Levels of gene flow, dispersal ability and whether populations occur in sympatry, can help to understand whether cryptic species are expected. Therefore, cryptic species are often morphologically similar taxa that either diverged in allopatry (and are currently free to disperse and come into following contact) (Stewart *et al.* 2010), or in sympatry (Jones & Van Parijs, 1993). Different climate changes, in the last 100k years have driven habitat availability and contemporary distributions of species and actual genetic structure (Hofreiter & Stewart, 2009). Reproductive isolation can separate populations either in allopatry, so that gene flow has no contact, or in sympatry, through resource

polymorphisms (Hoelzel, 1998). Furthermore, reproductive isolation between diverged populations may be incomplete upon secondary contact, leading to hybridization zones and introgression between the diverged lineages (Poelstra, 2014), potentially resulting in homogenization (Servedio & Noor, 2003), speciation by reinforcement (Hoskin *et al.* 2005), or possibly hybrid speciation (Amaral *et al.* 2014). In this framework, Pleistocene played a key role with rapid and dramatic climatic fluctuations, generating extensive environmental changes, which would have influenced the temporal and spatial distribution of taxa over glacial cycles (Hofreiter & Stewart 2009; Stewart *et al.* 2010). In the marine ecosystems, several changes have contributed to the spatial genetic structure and taxonomic variation in marine species. Oscillations in climate had dramatic effects on oceanographic processes such as temperature stratification and upwelling (Wang *et al.* 1999a) and determined patterns of isolation between areas (Gaither & Rocha, 2013).

The allocation of endangered species into particular 'stocks' or populations based solely on geographic boundaries seems illogical for most marine mammals given their huge potential for movement. Genetic analyses provide a more suitable means of assessing biologically significant population subdivisions. Significant subdivisions within and among populations seen via an examination of gene frequencies provides a fundamental tool for the management of exploited and protected species (Saura and Faria, 2011). The differentiation of gene frequencies within and among populations can be a result of gene flow via migration of individuals or their gametes, random genetic drift, natural and sexual selection modes, mutations, and genetic recombination opportunities that have been mediated by the mating system (Avise 1994). Female philopatry and male dispersal are the expected patterns of dispersion for mammalian species based on theoretical considerations (Greenwood 1980). The differences in dispersal between males and females may influence how populations are structured from a genetic perspective. Population structure affected by gender-based dispersal is particularly visible when one compares the haploid and maternally inherited mtDNA with the bi-parental nuclear genome (Avise 1994). If females are philopatric and males

disperse, it is expected to find more variation between putative populations with respect to mtDNA and less variation with respect to nuclear DNA. Previous cetacean studies on humpback whales (Baker *et al.* 1998), fin whales (Berube *et al.* 1998) and sperm whales (Lyrholm *et al.* 1999) using genetic techniques based on mtDNA and nuclear DNA provide valid support for this sex- biased dispersal scenario. In the ocean ecosystems there are fewer opportunities for allopatric divergence to occur, above all for highly mobile marine species where barriers to gene flow are relatively rare. Speciation and population structure are more likely to occur in sympatry or parapatry driven by environmental heterogeneity across space and time. In this framework, cetaceans disperse over relatively large distances and despite that, yet show significant genetic differentiation over relatively small spatial scales (see Tolley *et al.* 2001; Natoli *et al.* 2004; Natoli *et al.* 2008; Andrews *et al.* 2010; Fernandez *et al.* 2011; Hamner *et al.* 2012). The adaptation to local habitat features with the use of local resources, is reflected in cetacean population structure (Hoelzel, 1994). While Mysticetes (baleen whales) are generally solitary, several species of Odontocete (toothed whales) exhibit extreme social cohesion that is related to complex social structure, feeding strategies and real complex breeding systems and dynamics (Ross, 2001). Both environmental and social patterns have a key role in shaping the genetic structure observed in several Odontocete species. For examples, *Stenella longirostris* in the Hawaiian Archipelago, shows genetic structure consistent with habitat and resource availability associated with different islands (Andrews *et al.* 2010). Social cohesion and high relatedness between females within groups of *Stenella coeruleoalba*, contributes to significant genetic structure in the Mediterranean (Gaspari *et al.* 2007). The social cohesion and breeding system seen in North Pacific killer whales, *Orcinus orca*, enhances the genetic structure observed between killer whale populations (Pilot *et al.* 2010). Several cetacean species are long-lived, feed at high trophic levels and can exhibit long-term residency in coastal areas (Wells *et al.* 2004).

However, cetaceans are particularly vulnerable to environmental changes, for this reason the health and status of a population and the lower

trophic levels it depends on, reflect the natural and anthropogenic pressures on an ecosystem (Wells *et al.* 2004). Because of this, cetaceans have been proposed as sentinels for marine ecosystem health, variability, and degradation (Ross, 2000; Simmonds & Isaac, 2007; Moore, 2008; Bossart, 2011). Environmental changes, particularly those associated with habitat availability/distribution in space and time, are likely to impact on cetacean population structure.

1.13 Sperm whales and Population structure in Mediterranean

In the oceanic ecosystems, geographic barriers can play a key role in shaping genetic structure of cetacean populations. In general, baleen whales and sperm whales are highly mobile and possess the ability to move over incredible distances (Stevick *et al.* 2002), but migratory patterns may drive whales from different stocks to similar breeding and feeding grounds where mixing happens (Palumbi and Baker 1994; Larsen *et al.* 1996). For mammalian species, female philopatry and male dispersal are the expected patterns of dispersion based on theoretical considerations (Greenwood 1980). In sperm whale, several studies have given evidence for the geographic range over which males can affect genetic dispersal. Various types of data, such as dialects, genetics, mark-recapture data, morphology, parasitism and predation (Best 1979; Whitehead 1987; Whitehead and Amborn 1987; Amborn and Whitehead 1989; Rice 1989; Whitehead and Kahn 1992; Dufault and Whitehead 1995; Lyrholm and Gyllentsen 1998; Whitehead et al 1998; Lyrholm *et al.* 1999, Engelhaupt *et al.* 2009, Alexander *et al.* 2016) suggest philopatry among female sperm whales, while adult males are known to be capable of ranging over vast distances (Best 1979; Rice 1989; Whitehead and Weilgart 2000, Whitehead 2003).

Within Mediterranean, sperm whale population structure has not been completely and clearly assessed to date and many questions are still open. The relatively shallow and narrow channels of Sicilian straits and Sicilian channel that separate the western and eastern basins could be potentially barriers to

movement for a species that has a strong preference for deep water as sperm whale. Still it is not well confirmed if there is any population structure within the Mediterranean and if the low gene flow, mediated by males coming from Atlantic through Gibraltar straits, is affecting/driving structure in our basin. If there was, this would make the conservation status of the two populations (western and eastern, or others) more and more precarious. Photo-id studies showed evidence of movements within the Western Mediterranean (as discussed in paragraph 1.1) but whether and how breeding happens is still unclear. Actual knowledge strongly supports the idea of wide movement of sperm whales within western basin, although many data from north Africa are still missing (Rendell and Frantzis, 2016).

Among scientists, rised the hypothesis of whether Mediterranean sperm whale population is divided in the eastern and western basins, as found for other cetaceans (like bottlenose and striped dolphins; Natoli *et al.* 2005, Gaspari *et al.* 2007, 2013). To assess this point, interesting evidences come from photo-id results after a mass stranding event of seven sperm whales that a happened in Adriatic Sea in December 2009 – as mentioned in *Stranding events* paragraph of this thesis. These whales were all males and stranded alive, but all died during the following two days (Mazzariol *et al.*, 2011). Photo comparisons of the flukes of stranded animals were made against catalogues from the whole Mediterranean. The following picture (fig 1.14.1) and table (tab 1.14.1) have been extracted from Frantzis *et al.* (2011). The results on the movements of three of the seven stranded individuals are well summarized in this table and map.

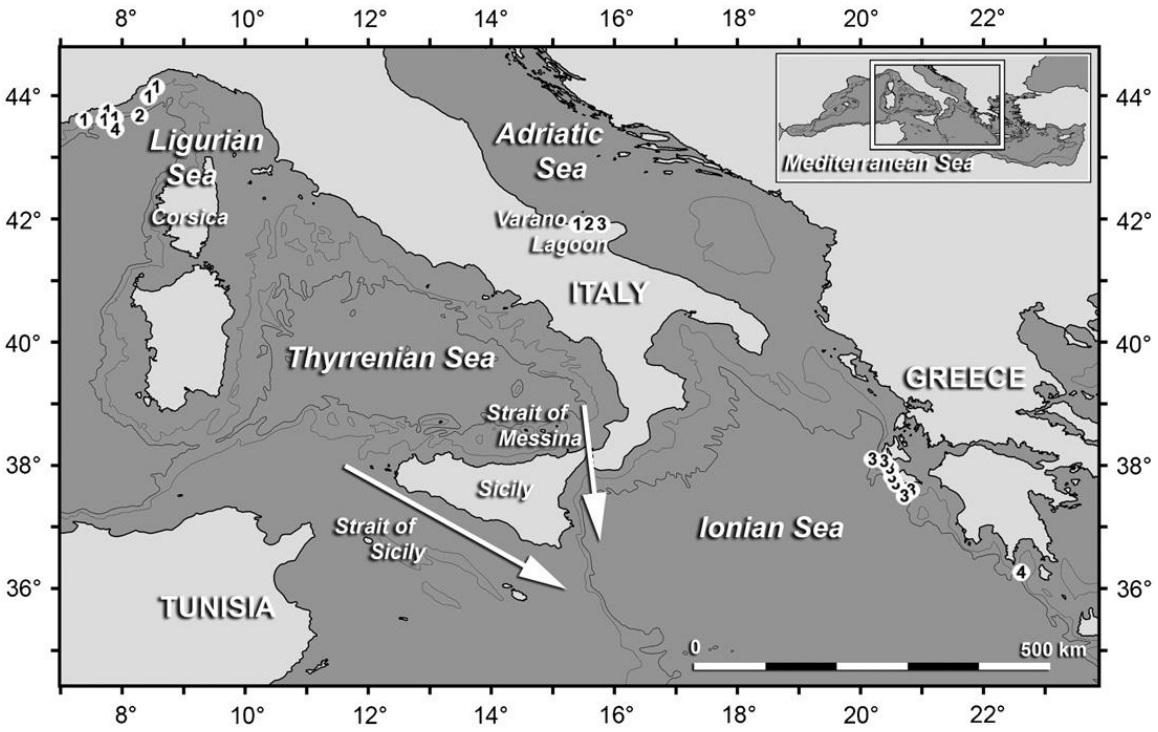


Figure 1.13.1: Maps of inter-basin movements in Mediterranean Sea. Observations of the whales identified in both the western and eastern Mediterranean basins and/or identified in the mass stranding are shown by numbered white dots. 1: CLA, 2: POMO, 3: ZAK WHITEHEAD, 4: ODYSSEAS. Figure from Frantzis et al. (2011)

Whale name	Sex	Age	Number of encounters	Date	Geographical area	Mediterranean basin	Encounter type	Number of individuals
CLA (#6)	Male	20–21	7	6 Jul 2002	W Ligurian Sea	Western	Solitary male	1
				5 Aug 2003	W Ligurian Sea	Western	Male aggregation	2
				21 Aug 2003	W Ligurian Sea	Western	Male aggregation	4
				27 Aug 2003	W Ligurian Sea	Western	Male aggregation	5
				6 Jul 2005	W Ligurian Sea	Western	Male aggregation	2
				12 Aug 2007	W Ligurian Sea	Western	Male aggregation	3
				11 Dec 2009	SW Adriatic Sea	Eastern	Male mass stranding	7
POMO (#2)	Male	19–20	2	30 Jul 2003	W Ligurian Sea	Western	Male aggregation	4
				11 Dec 2009	SW Adriatic Sea	Eastern	Male mass stranding	7
ZAK WHITEHEAD (#5)	Male	15	9	5 July 2000	SE Ionian Sea	Eastern	Social unit	≥5
				27 Aug 2002	SE Ionian Sea	Eastern	Social unit	12
				24 Jul 2005	SE Ionian Sea	Eastern	Social unit	7
				26 Jul 2005	SE Ionian Sea	Eastern	Social unit	7
				27 Jul 2005	SE Ionian Sea	Eastern	Social unit	7
				31 Jul 2005	SE Ionian Sea	Eastern	Social unit	7
				2 Aug 2005	SE Ionian Sea	Eastern	Social unit	7
				31 Aug 2005	SE Ionian Sea	Eastern	Social unit	7
				11 Dec 2009	SW Adriatic Sea	Eastern	Male mass stranding	7
ODYSSEAS	Male	n.a.	2	3 Aug 1991	W Ligurian Sea	Western	Solitary male	1
				6 Aug 2004	W Kythira Sea	Eastern	Solitary male	1
				7 Aug 2004	W Kythira Sea	Eastern	Solitary male	1

Table 1.13.1: Details on photoID recaptures within Mediterranean Sea. Table from Frantzis et al. (2011).Data on all the observations of the three stranded whales that matched with previously observed free-ranging sperm whales (CLA, POMO and ZAKWHITEHEAD) and the free ranging whale that was photo-identified in both Mediterranean basins (ODYSSEAS).

These have been the main findings according to Frantzis *et al.* (2011):

- three of the seven had been previously identified, two in the western Basin and one in the eastern Basin.
- The male that did not change basins had been seen several times along the Hellenic Trench, first in 2000 as a juvenile member of a social unit, likely his natal unit, and seven more times in 2002 and 2005, always with the other members of this social unit. In 2009, this animal measured 10.5 m and tooth sectioning resulted in an age estimate of 15 years.
- Two other individuals from the stranded group had previously been observed and photo-identified in the western Mediterranean, specifically the north-western Ligurian Sea. One individual (12.1 m long and 20–21 years of age) was firstly photo-identified in 2002, and observed five more times in 2003, 2005 and 2007, before stranding in 2009, and the other (12.2 m long and 19–20 years of age) was first identified in 2003, but not seen again until the stranding event.

These sperm whales followed a route from the northwest Ligurian Sea to the stranding location of about 2000 km, and obviously crossed either the Strait of Messina or the Strait of Sicily. Thus, male sperm whales originating from both the eastern and western basins joined and formed a group in the eastern basin – but there is no evidence of reproductive success. A final important matching – not involving the stranded individuals mentioned above - was that of a male sperm whale, which moved from the western to the eastern Basin. This whale was photographed in the western Basin in 1991 for the first time and was re-captured along the Hellenic Trench in the eastern Basin in 2004: In the tab 1.14.1 its name is ODYSSEAS (Frantzis *et al.*, 2011).

Consistent with the photo-identification results described above, the stable isotopes analyses results gave important findings. A sharp shift in teeth growth layer group $\delta^{15}N$ and $\delta^{13}C$ isotope levels was observed by Mendes *et al.* (2007) from a stranded male. This shift corresponds to 20 years of age, when in the oceanic populations male sperm whales make large migrations from feeding to breeding grounds (Whitehead, 2003; Whitehead and Weilgart,

2000). These results demonstrate also a significant change in dietary sources: considering the well-known variations in isotope levels between the eastern and western basins, Mendes *et al.* (2007) results suggested a male movement from the western to the eastern Mediterranean regions. This result strongly supports the hypothesis of West-Est movement described in Frantzis et al (2011). However, the evidence we have up to now, all comes from males, it is unclear whether such movements are performed by females too (Rendell and Frantzis, 2016).

In the framework of conservation genetic, these findings of gene flow male mediated – if present - could be enough to stop the populations from diverging. Without those connections, we would be considering two smaller and consequently even more vulnerable populations, rather than a single vulnerable one. But a detailed genetic assessment to proof an effective gene flow through Sicilian waters is still missing. If it was confirmed, keeping the east–west population links open is a key point in order to insure the long-term viability of the population. The potential impact of any human activities in the Straits of Messina and Sicilian channel need to be monitored because it could alter these movements (Rendell and Frantzis, 2016).

1.14 Sperm whale molecular ecology

In the last 20 years, sperm whale population genetic has been investigated with different methodologies at global and regional scale.

Lyrholm *et al.* (1996) started sequencing mtDNA control region from **37** sperm whale, sampled in North Atlantic, North Pacific, Galapagos Islands, Southwest Pacific and Antarctic, and identified 13 mtDNA haplotypes. The low mtDNA diversity assessed, indicated a young global population structure with an age of less than ca. 100k years, perhaps even less than 25k years. This may reflect an expansion to the actual range after glaciations of the Pleistocene period, when suitable habitats could have been restricted and ocean circulation patterns could have been changed (McCabe & Clark 1998), affecting the availability of sperm whale preys. It has been proved that ocean cooling and warming influenced the distribution and abundance of many cetacean species (Gaskin 1982). Thus, Pleistocene glaciation could have provoked a restricted distribution of the sperm whale, particularly of females and young, which normally do not range into cold waters (Best 1979), and food availability may have been seriously diminished.

Subsequently, Lyrholm and Gyllensten (1998) analysed **231** individuals again from three major oceanic regions, the North Atlantic, the North Pacific and the Southern Hemisphere at the first 330 base pairs in the mitochondrial DNA and identified 16 mtDNA haplotypes. In this study, the results were consistent with Lyrholm *et al.* (1996): the most common mtDNA types were present in more than one oceanic region, but ocean-specific types were rare. Genetic differentiation was found between potential social groups indicating matrilineal relatedness within groups.

Lyrholm *et al.* (1999) extended the analyses on nuclear DNA. No significant heterogeneity in allele frequencies of microsatellite loci was found. These contrasting patterns with Lyrholm and Gyllensten (1998) suggest a sex biased dispersal with interoceanic movements driven by males, and philopatry of females to their natal area.

Ten years after, Lyrholm *et al.* findings were supported by Engelhaupt *et al.* (2009) results, analysing mtDNA control region and 16 polymorphic microsatellite loci, from samples of Gulf of Mexico, western North Atlantic, Mediterranean Sea and North Sea. In this study, Gulf of Mexico, western North Atlantic and North Sea populations revealed same low levels of haplotype and nucleotide diversity at the mtDNA locus, while the Mediterranean Sea population showed no detectable mtDNA diversity – just one haplotype. From mitochondrial DNA results, significant differentiation between all populations has been detected, while microsatellites showed significant differentiation only for comparisons with the Mediterranean Sea, and at a much lower level than seen for mtDNA. This study provided new structuring evidence of populations within an ocean and among adjacent seas. The two major coastal basins on either side of the North Atlantic are home of philopatric populations with matrifocal social groups, from which males disperse, probably on an oceanic scale.

Mesnick *et al.* (2011) used 400 bp from mtDNA, six microsatellites and 36 SNPs to investigate population structure of sperm whales in the eastern and central North Pacific. In the temperate waters where females are, three strata have been estimated: California Current, Hawai`i and the eastern tropical Pacific. The most interesting result was the differentiation of California Current stratum from the other ones at mtDNA, microsatellites and SNPs, suggesting that in the region, there is a demographically independent population with males reproductive philopatry. Comparisons with Alaska males were statistically significant from all three strata and individuals showed mixed assignment suggesting widespread origin of males on sub-Arctic feeding grounds. These results showed genetic differentiation in the eastern Pacific Ocean, "where no physical barriers exist among geographically defined strata", and contrast with previous results in the Pacific Ocean (Lyrholm & Gyllensten 1998; Lyrholm *et al.* 1999).

Alexander *et al.* (2013) listed three possible hypotheses as the most likely causes of the low mtDNA diversity in sperm whales:

- a population bottleneck and/or expansion as proposed by Lyrholm *et al.* (1996) and Lyrholm & Gyllensten (1998).
- a selective sweep due to a favourable substitution in a mtDNA-encoded protein (see Janik 2001).
- a selective sweep due to beneficial cultural traits transmitted matrilineally in parallel with the mitogenome – cultural hitchhiking as described in Whitehead (1998; 2005).

Again, the same author (in Alexander *et al.*, 2016) combined mtDNA information for 1091 previously published samples with 542 newly obtained DNA profiles (394-bp mtDNA, 13 microsatellites). She hypothesized that the low but highly structured mtDNA diversity, observed in sperm whale, is consistent with a recent, rapid radiation of a single mtDNA lineage, followed by genetic drift due to female philopatry at regional and social group levels. The estimated expansion began about 80k years ago, during last glacial maximum (Lambeck *et al.*, 2014). An interesting and parallel finding was the low mitogenomic diversity of one of the main sperm whale's prey (Winkelmann *et al.* 2013): the giant squid (Architeuthis spp.). This raises the possibility that a worldwide expansion of sperm whales could have been predicated on a recent expansion of their prey, especially as other squid species have also shown signatures of demographic/range expansions that appear to be associated with the last glacial maximum (e.g. *Dosidicus gigas*, Ibanez *et al.* 2011; *Doryteuthis gahi*, Ibanez *et al.* 2012; Ibanez & Poulin 2014). In contrast with the high levels of maternal structure found at various hierarchical scales, nuclear structure was far less pronounced because of male biased gene flow.

Warren *et al.* (2017), to better understand patterns of genetic diversity among sperm whales from different ocean basins, carried out a medium-coverage resequencing of individuals from the Pacific Ocean and Indian Ocean. Average genome-wide heterozygosity per base, corrected for callable sequence space, was 0.0011. This value is low in comparison with the fin whale (0.0015) and bottlenose dolphin (0.0014; Yim *et al.* 2014), suggesting the sperm whale has a smal effective population size (Ne). A pairwise sequentially

Markovian coalescent (PSMC) analysis (Li and Durbin, 2011) indicated a rapid decline in Ne during the transition from the Pliocene to Pleistocene epoch (fig. 1.15.1).

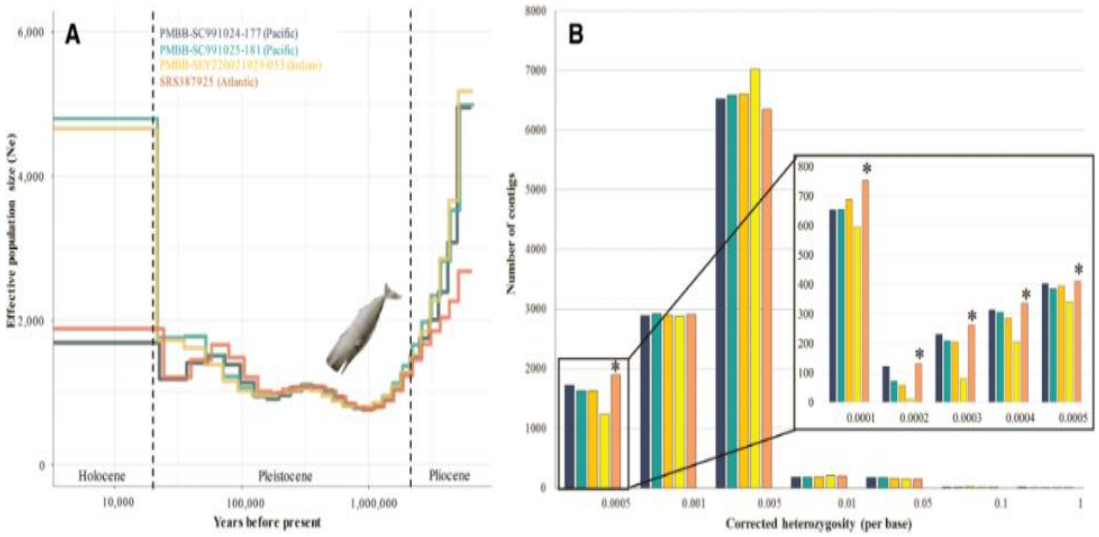


Figure 1.14.1: PSMC demography graph. From Warren et al. 2017
Warren, W. C., Kuderna, L., Alexander, A., Catchen, J., Pérez-Silva, J. G., López-Otín, C., ... Wise, J. P. (2017). The novel evolution of the sperm whale genome. Genome Biology and Evolution, 9(12), 3260–3264.

Contemporary, Morin *et al.* (2018) analysed 175 globally distributed mitogenomes and three nuclear genomes to test hypotheses about population bottleneck and expansion vs. a selective sweep due to cultural hitchhiking or selection on mtDNA as possible factors that contributed to low worldwide mitochondrial diversity in sperm whales. These results show that mitogenome haplotypes are ocean-specific: 65 of the 80 haplotypes were found only in the Pacific, 14 were found only in the Atlantic, and only one haplotype (mt03) was found in both ocean basins. Demographic analyses of nuclear genomes suggest low mtDNA diversity is consistent with a global reduction in population size that ended approximately 125,000 years ago, correlated with the Eemian interglacial. Analysis on phylogeography suggests that sperm whales descend from maternal lineages endemic to the Pacific that have subsequently colonized the Atlantic in several events and a recent expansion (20–40 kya), with female movement among oceans only in warmer period (fig. 1.15.2).

Lastly, Fan *et al.* (2019) mapped the sequencing data of five sperm whales to reference genome and identified ~8.47 million SNPs in total, with a diversity level of 0.00136, comparing 0.0009 of killer whale (Foote *et al.*, 2016) and 0.0008 pf finless porpoises (Zhou *et al.*, 2018). The population demography has been inferred using PSMC, that showed a similar population history to that illustrated previously (Warren *et al.*, 2017) with a rapid decline in population size during the Pliocene to Pleistocene transition and increases thereafter (fig. 1.15.3).

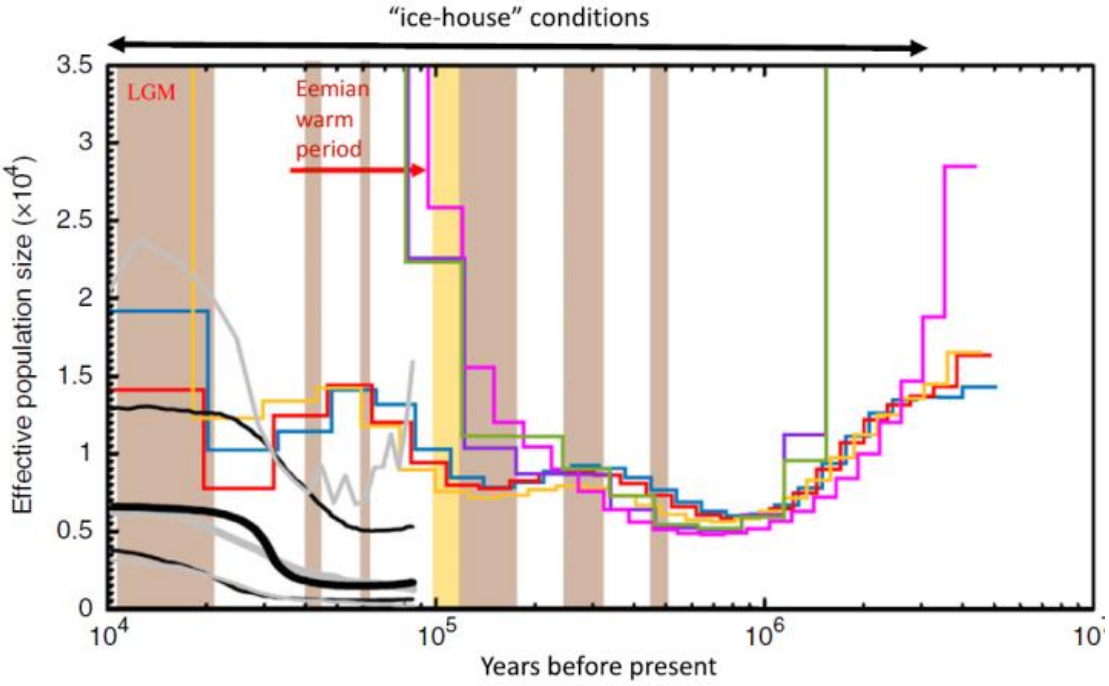


Figure 1.14.2: PSMS demography graph.
Atlantic (red), Pacific (blue) and Indian (yellow) Oceans. Pseudodiploid plots are shown for each ocean pair: Atlantic–Pacific (green), Atlantic–Indian (pink) and Pacific–Indian (purple). From Morin, P. A., Foote, A. D., Baker, C. S., Hancock-Hanser, B. L., Kaschner, K., Mate, B. R., ... & Alexander, A. (2018). Demography or selection on linked cultural traits or genes? Investigating the driver of low mtDNA diversity in the sperm whale using complementary mitochondrial and nuclear genome analyses. Molecular ecology, 27(11), 2604-2619.

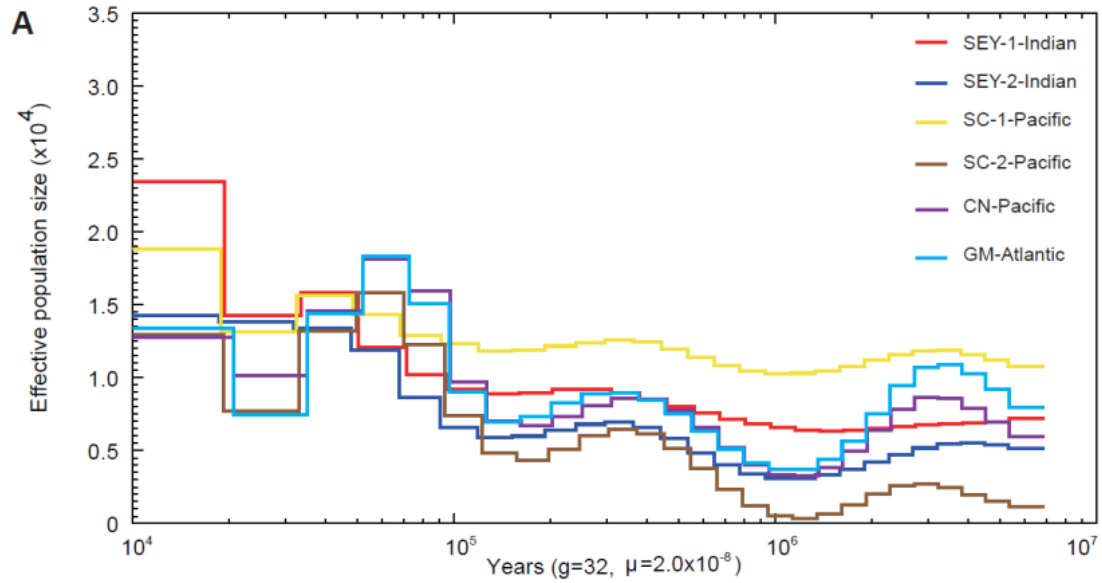


Figure 1.14.3: PSMC demography graph.
From Fan, G., Zhang, Y., Liu, X., Wang, J., Sun, Z., Sun, S., ... & Tan, X. (2019). The first chromosome-level genome for a marine mammal as a resource to study ecology and evolution. Molecular ecology resources.

Summarily:

- mtDNA diversity in sperm whales, is relatively low within basins and oceans (Lyrholm *et al.* 1996; Alexander *et al.* 2016) and from moderate to high among oceans (Lyrholm & Gyllensten 1998), among marginal seas within the Atlantic (Drouot *et al.* 2004; Engelhaupt *et al.* 2009) and among social groups within the Pacific (Lyrholm & Gyllensten 1998; Rendell *et al.* 2012).
- both in Atlantic and in Indian Ocean, marked patterns of maternal structure at regional scale have been found despite the absence of marked geographic boundaries (Alexander *et al.* 2016).
- In contrast, in Pacific Ocean not marked genetic structure at mtDNA has been detected (Rendell *et al.* 2012; Mesnick *et al.* 2011). The lack of regional structure in this ocean is consistent with behavioural evidence: that is, females dispersal up to 4000 Km, while in the Atlantic Ocean they range only up to 700 km (Jaquet *et al.* 2003; Whitehead *et al.* 2008, 2012; Ortega-Ortiz *et al.* 2012; Mizroch & Rice 2013). Acoustically, in Pacific Ocean clans are sympatric (Rendel *et al.* 2012) rather than allopatric as seen in Atlantic

Ocean (Whitehead *et al.* 2012). So, the lack of geographically mtDNA differentiation in Pacific Ocean is explained by a maternal dispersal at wider geographic scale than Atlantic. Social group was the only level that explained any significant amount of genetic variation in this ocean.

- The different picture described by mtDNA and nuDNA results has been interpreted as **male dispersal** and **female philopatry** at oceanic scales, between regions within the same ocean and at the social group level. These findings agree with behavioural dynamics of sperm whale social structure: at 3–15 years old males disperse from their natal social units (Best 1979; Richard *et al.* 1996; Whitehead 2003), and as they age choose a solitary life and reach polar waters of the oceanic basins for feeding on more energetic fish preys (Best 1979; Allen 1980; Whitehead 2003). At 25–27 years, reached the social maturity (Best 1979), solitary males are found associated with females for the purposes of mating, but, as observed in others mammals, they do not permanently remain with female social group (Whitehead 1993, 1994; Richard *et al.* 19963). Female social groups are composed of adult females with long-term social bond and juveniles of both sexes. These social groups remain confined to low latitude tropical and temperate waters (Best 1979; Richard *et al.* 1996a; Christal *et al.* 1998; Dufault & Whitehead 1998; Dufault *et al.* 1999; Coakes & Whitehead 2004).

Within the Mediterranean Sea, three studies have given partial results on population structure in this area. But many questions are still open. Initially, Drouot *et al.* (2004) used 13 samples from the western and eastern Basins and sequenced the first 200 bp of the mitochondrial DNA control region and compared them with sequences of stranded animals from the coasts of the British Isles and northern Europe. The most important finding was that all 13 sequences from Mediterranean shared the same haplotype, found in 28 of the 57 sequences from Atlantic. These results reflect different maternal structures between the two areas and the differentiation in mtDNA haplotypes is consistent with the behaviour of female sperm whales, which show site fidelity.

Subsequently, Engelhaupt *et al.* (2009) as described before, analysed 301 sperm whale tissue samples from several locations and 44 of these were collected in Mediterranean Sea. He assessed variation in both mtDNA (up to 399 bp) and 16 nuclear microsatellite loci. About mtDNA, he found that all the Mediterranean samples shared an identical mitochondrial sequence, which they identified as haplotype 'C' - not present just in Mediterranean, but it is one of the three most common sequences in sperm whales, and it was also found in the eastern tropical Pacific (Rendell *et al.*, 2012). At nuDNA, microsatellites showed significant differentiation but much lower level than seen for mtDNA. These results demonstrated a fidelity of females to basins such as Mediterranean and Gulf of Mexico and suggest the movement of males among these areas for breeding purposes.

More recently, Alexander *et al.* (2016) also found just a single mtDNA haplotype among 40 individuals sampled within Mediterranean.

Last study that included some samples from Mediterranean Sea was from Morin *et al.* (2018). Using for samples found two haplotypes (see tab S7 in Morin *et al.*, 2018), previously identified as a single haplotype (haplotype C). However, the birth of Mediterranean lineages corresponds to 20k years ago, that is the end of last glacial maximum (see fig 2 and 5 in Morin *et al.*, 2018).

1.15 Mediterranean Sea

The Mediterranean is a large sea located between southern Europe and northern Africa, around the 30°N and 45°N, and 6°W and 36°E. It extends over 3000 km in longitude and over 1500 km in latitude and has a surface area of more than one million square kilometres (fig. 1.16.1)

Figure 1.15.1: Mediterranean Sea
Screenshot from NASA World Wind

It is an almost totally land-locked sea, joined to the Atlantic, through a single, relatively narrow entrance at the Strait of Gibraltar. The maximum depth of the sea is about 5125 m, in the Ionian Sea and its floor is made up of a complex system of ridges, troughs and deep basins. Compared to larger oceans, its continental shelf is extremely narrow and deeply incised by submarine canyon systems. The two narrow channels of Straits of Sicily (250 m deep) and Sicilian channel (316 m deep) divide the Mediterranean in two distinct basins eastern and western (Nielsen 1912) (fig 1.16.2). The Western Basin is characterised by broad, generally smooth abyssal plains. In contrast, the Eastern Basin is dominated by the Mediterranean ridge system and has a much lower proportion of smooth abyssal plain (Truver, 1980). This sea is famous for its unique water circulation, driven by the connection with the Atlantic ocean

that combined with the topography of the bottom and the shape of the coasts, determine oceanographic features: high salinity (36-38‰), strong seasonal fluctuation of the water temperature, scarce upwelling – except few regions - and the non-homogenous distribution of nutrients (Marullo *et al.*, 1994).

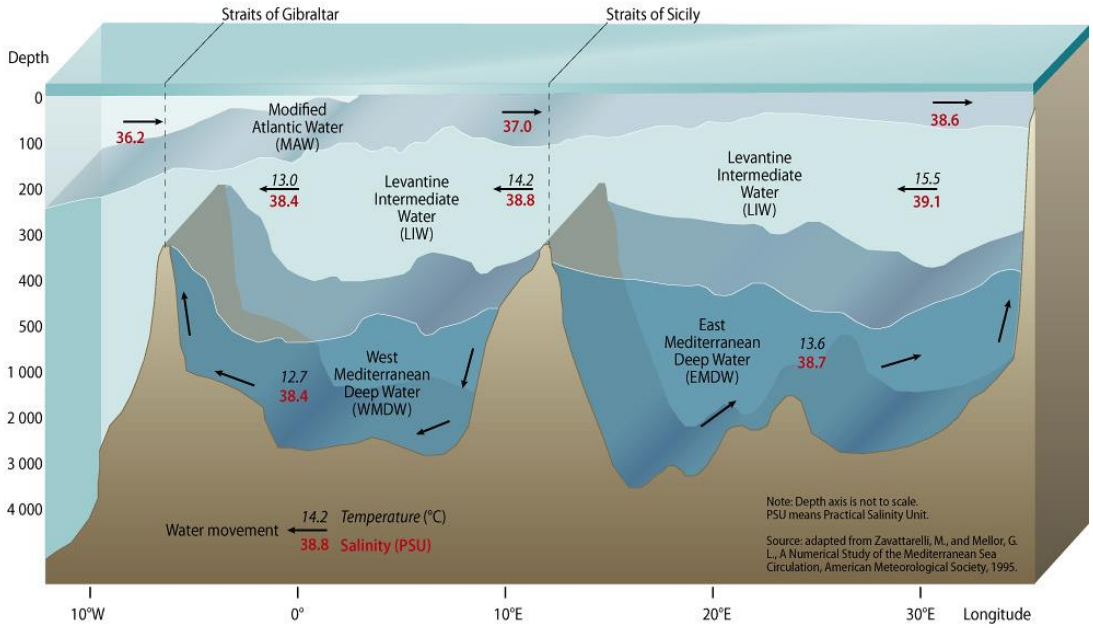


Figure 1.15.2: vertical distribution of water masses in Mediterranean Sea.

Thus, the Mediterranean is an oligotrophic sea with low levels of primary production. However, some areas show an increasing of productivity locally and seasonally.

The Western basin is heterogeneous (Jacques & Treguer 1986): regions located above the 41° parallel appear to be relatively rich, with high levels of primary production, from 0.5 to 1 mg of Chla per m^3 in the Gulf of Lions and about 0.4 mg of Chla per m^3 in the Ligurian Sea. The Gulf of Lions is one of the few areas remaining mesotrophic throughout the summer (Millot 1979, Morel & André 1991). The general current flowing to the west along the continental slope of Provence, Gulf of Lions and into the Balearic Sea, contribute to the dispersal of the food chain westward from the Ligurian Sea frontal system, known as a permanent source of primary production (Prieur 1981). This food chain drift feed higher trophic level organisms, suitable to top predators as

cetaceans. The Tyrrhenian Sea is commonly considered as a distinct entity, because it is semi-enclosed between islands (Corsica and Sardinia) and mainland (Italy), and separated from the rest of the western basin by a channel of moderate depth, ca. 1500 m. It is characterised by a marked oligotrophy throughout the year.

The eastern basin is considered to be one of the most oligotrophic areas of the world with extremely low levels of chlorophyll a concentration (Psarra *et al.*, 2000). The continental slope and bathyal sediments of the eastern Mediterranean are characterized by extremely low concentrations of potentially limiting organic nutrients (e.g. proteins and lipids) that sharply decline with increasing distance from the coast and sediment depth (Tselepides *et al.*, 2000). In this area, the Hellenic Trench runs parallel to the western, southern and south-eastern coasts and islands of Greece. It is 1100 km long and characterized by a series of linear Trenches and small troughswith steep relief and the 1000 m contour typically within 3–10 km from the mainland. This area is probably the most important point in the eastern basin, acting as feeding and breeding area for marine mammals' deep divers.

1.16 Aims of this project

Sperm whales spend most of their time underwater where it is impossible for researchers to observe their behaviour. The population structure is a central point of molecular ecology and conservation biology. An understanding of pattern of connectivity and of ecological forces that drive their dispersal and isolation are a key point for conservation management planning. In this framework, the presented project will apply a high-resolution genome sampling method (the double-digest RAD method; Peterson *et al.* 2012) to obtain a detailed assessment of Mediterranean sperm whale population genetics. This method uses restriction enzymes (which cut DNA at predictable sites) to fragment DNA. Adapter constructs are then legated onto these fragments and massively redundant parallel sequencing (on an Illumina HiSeq 2500) can then be applied to generate ~700MB of sequence reads per sample, enough for the identification of ~5000 SNP loci. This level of resolution will permit highly accurate assessments of parentage and kinship (e.g. Konovalov *et al.* 2004, Anderson and Garza 2006), the degree of historical or recent connectivity among populations (e.g. Wilson and Rannala 2003, Pickrell and Pritchard 2012) and the effective size and demographic history of a population (e.g. Storz and Beaumont 2002, Hare *et al.* 2011).The hypothesis of sub-structure in the population of sperm whales within the Mediterranean Sea will be tested. Given evidence for substructure, we will test for directional gene flow among sub-populations, evidence of ongoing gene flow, assess the effective size of sub-populations, and determine patterns of historical demographics. These measures will inform us about the level of risk in each sub-population and the potential for recovery (associated with the level of isolation, inbreeding, and demographic trajectory). Tests for kinship within and among groups will extend research undertaken elsewhere in the Atlantic where kin associations within groups were found to be weak (i.e. Ortega *et al.* 2012).

Understanding the ecology and the pattern of connectivity of the Mediterranean population are crucial to appropriately designing and

implementing measures to conserve it. The Mediterranean Sea is closed off from the world's oceans except for the narrow Strait of Gibraltar. Rendell and Frantzis (2016) raised two critical questions about this population:

- how much is it isolated from the larger populations of Atlantic?
- Does it represent a 'lost tribe', descendants from a single colonisation event that has since remained isolated on its own trajectories, or are they an extended 'lobe' of the huge North Atlantic population?
- Is this population divided in any isolated reproductive stocks?

The answers to these questions will strongly impact the conservation plans we should take for the Mediterranean sperm whale: high degree of isolation will lead to greater risk of biodiversity loss, and with an increase of human impact and without a replenished from neighbouring Atlantic stocks, the population will be highly vulnerable.

2 Materials and Methods

2.1 Samples collection

Initially, for this study, 240 sperm whale tissue samples in total were provided from 10 different geographical regions within the Mediterranean and the outdoor close area of North Atlantic Ocean. Mediterranean samples were collected from 6 different areas in 4 different countries (Greece, Italy, France, Spain) Atlantic samples were provided from 4 different areas in 3 different countries. In the following table are all samples provided:

Area	Biopsy from stranding	Biopsy from free ranging	Sloughed skin	Total	Samples used for this study
Greek Seas [1]	*21*	-	*69*	90	21
Adriatic Sea [1]	*11*	-	-	11	11
Tyrrenian Sea [1]	*4*	-	-	4	4
Messinian strait [1]	*1*	-	-	1	1
Ligurian Sea [1]	-	*74*	-	74	65
Balearic Sea [1]	-	*6*	*2*	8	8
Gibraltar Strait [1]	*2*	*18*	-	20	10
Canarias [2]	*15*	-	-	15	11
Madeira [2]	-	*12*	*2*	14	13
Azores [2]	*2*	*11*	*7*	19	12
North Spain [2]	5	-	-	5	4

Table 2.1.1: samples provided from each area.
[1] Mediterranean areas, [2] Atlantic areas

Biopsy samples were collected from free-ranging whales using a sterilized corer tip attached to a dart fired from a crossbow and stored in ethanol 95% or salt /DMSO (Amos & Hoelzel 1991). Archived material was also obtained from stranded whales in different areas. Of 240 samples 160 samples gave positive

results in DNA quality and quantity and have been used for the chosen protocol.

2.2 DNA Extraction

Whole-cell DNA was extracted by kit (OMEGA BIOTEK and MN MACHEREY-NAGEL) following the manufacturers protocol and phenol chloroform method (after Hoelzel 1992). Standard phenol-chloroform DNA extraction protocols, as adapted from Hoelzel (1998b), were carried out on tissue samples. Approximately 100 mg of tissue was finely chopped and added to 500 µl of extraction buffer (50 mM Tris pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% (w/v) SDS). A further 45 µl of proteinase-K (10 mg/ml) was added to the solution and the tissue was left to digest overnight in a water-bath at 37°C with occasional agitation. 500 µl of phenol was added to the digestions, mixed thoroughly, and then centrifuged for 5 min at 7000 x *g* to separate the phases. The surface aqueous phase was pipetted off and transferred to a new tube while the organic layer took no further part in the extraction process and was appropriately discarded. This process was repeated a second time with phenol and then with a mixture of phenol:chloroform:isoamyl-alcohol (25:24:1 by vol.). Using chloroform:isoamyl alcohol (24:1 by vol.) the process was repeated once more and the final separated aqueous phase was transferred to a new tube. Subsequently, 0.1 vol. (~45 µl) of 3M sodium acetate was added and mixed 1 ml of chilled 100% ethanol was then added to precipitate the DNA and put in a freezer to incubate at -20°C for approximately 1 hr. Once precipitated, the DNA was centrifuged at 7000 x *g* for 15 min to pellet the DNA. The supernatant was removed and replaced with chilled 70% ethanol and centrifuged again to clean the DNA pellet. The supernatant was removed, and the DNA pellets were dried in a centrifugal evaporator. DNA was re-suspended in an appropriate volume (~200 µl) of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). To perform RADs, the extracted DNA has been run on electrophoresis gel to assess the fragments length and quantified by Qubit machine using DNA high sensitivity KIT. DNA with HMW and with concentration higher than 10 ng/uL has been chosen for the presented study.

In detail the samples used have been; 12 from Azores, 11 from Canarias, 13 from Madeira; 4 from North Spain; 10 from Strait of Gibraltar, 8 from Balearic islands, 60 from Ligurian Sea, 3 from Tyrrenian Sea; 11 from Adriatic Sea and 21 from Greece.

2.3 Restriction enzyme digestion

The enzymes used in my analyses are MspI and HindIII-HF provided by NEBengland.

2.4 Adapter ligation

Adapter mix (15uL) and digested DNA (45uL) was combined for adapter ligation step. The PCR was run with the following program: 22°C for 2 hours, 65°C for 20 min, 8°C forever. As soon as the program reaches the last stage (8 degrees forever), the samples have been taken out and proceed to the pooling step.

2.5 First pooling

In this step, 16 pools have been done (8 pools per lane, considering I have two lanes). In each pool are ten samples with different adapters (from I to X, from 12 adaptors that are in our lab, I used 10 of them). From previous step, I had 60 uL per samples, so now, are 60 uL x 10 = 600 uL in each pool.

2.6 Purification by Beads

Each pool of 600 uL has been divided in two tubes with 300 uL each. It's important to do this parting in order to perform the most efficient bead cleaning using Sera-Mag SpeedBeads.

2.7 Quantification

At this stage, samples have been quantified in order to know the percentage of DNA lost during the beads cleaning step. Quantification has been done by Qubit as previous described.

2.8 Size selection by Pippin prep

At this step, a size selection Sage Science PippinPrep machine has been performed. From each pool, fragments between 360 and 560 bp has been selected for further analyses. From each sample, 30 uL have been used. In the end, the elution has been per formed in 40 uL. Because of exclusion of fragments out of the chosen windows, the final volume has got the lowest concentration of the entire protocol.

2.9 Indexing (barcoding the pools)

The Pippin Prep output of 40 uL DNA sample per pool has been splitted in 4 samples of 10 uL to perform the PCR reaction. Selected fragments have been amplified, using using Phusion High-Fidelity PCR Kit (which contains Taq polymerase).

2.10 Purification by column

At this stage, each pool that has been divided in 4 volumes of 10 uL to perform PCR amplification, has been repolled and cleaned with Invitrogen DNA Purification Kit. At the end of this stage, the elution phase has been conducted with 30uL of Eb (40-50 uL).

2.11 Quality control

To confirm the positive reaction of the last PCR, a new Qubit quantification has been performed to assess the new concentrations values after amplification by Phusion. Here the concentration increases from 30 to 100 times. At this stage is mandatory to be sure that each pool has the right quantity of DNA and the right fragment length. These checks have been performed as described in the next two paragraphs.

2.12 Tape Station

The tape station has given confirm about the fragment length (360-560 bp as described in Pippin Prep step 2.8) and some values on concentration in nM. In performing the Tape Station, 2 uL of the cleaned product containing 1 ng of DNA have been combined with 2 uL of high sensitivity buffer.

2.13 qPCR

The real time tine PCR is a technique able to give the most accurate output about concentration of each pool. The standard used have been 20pM, 2 pM, 0.2 pM and 0.02 pM. In order to have concentrations values in the range of the standard, two sets of dilutions have been performed 1:40000 and 1:60000. Two replicates for each pool in the two dilutions have been performed. The standards have been triplicates respectively. The reaction has been run using qPCR Mastermix (2x KAPA SYBR qPCR mix universal).

2.14 Pooling round number 2 and Illumina sequencing

All pools have been now rebalanced to concentration of 10 nM. In the end 8 uL of each pool (from A to H and from I to P) have been pooled in two final pools. Libraries were paired-end sequenced on an Illumina HiSeq_2500 (version 4 chemistry) and sequenced at Durham University's DBS genomics service.

2.15 Sex determination

For each skin biopsy and sloughed skin samples, 103 and 2 respectively, gender was determined amplifying ZFX/ZFY regions, as described in Berube and Palsboll (1996). Samples collected in stranding events, with known sex, have been used as a means of confirmation for PCR amplifications and yielded expected results.

3 Data Analyses

3.1 SNP calling, filtering and data management

Reads were trimmed to 110 bp and demultiplexed and filtered using STACKS2.2 (Rochette *et al.*, 2019). Unpaired reads were discarded. Paired reads were aligned using the very-sensitive mode of Bowtie2.2.5 (Reinert *et al.*, 2015) against a sperm whale reference genome downloaded from the NCBI website (GCA_002837175.2 https://www.ncbi.nlm.nih.gov/assembly/GCF_002837175.2). Samtools version 1.9 (Li *et al.*, 2015) was used to filter out reads which aligned disconcordantly, reads with a mapping quality below 20, as well as reads which aligned to more than one location in the genome. SNPs were called using the STACKS refmap pipeline with default settings. The populations command of STACKS was run with r=0.7 and p=3. We accepted multiple SNPs per read (i.e. we did not set the –write-single-SNPs flag), as we opted to optionally 'thin' our dataset downstream. PGD-Spider (Lischer and Excoffier, 2012) or vcftools (Danecek *et al.*, 2011) was used to convert the SNP data into PED and MAP format. Binary files (BED, RAW and BIM) were generated from PED and MAP files using PLINK (Purcell *et al.*, 2017). The vcftools (Danecek *et al.*, 2011) flags --depth and --site-depth were used to calculate read depth per individual and per SNP. SNP data management and analyses were performed in R-3.6.2 (R Core Team, 2019) using wrapper functions of the R package SambaR (https://github.com/mennodejong1986/SambaR). The data was imported into R and stored in a genlight object using the function 'read.PLINK' of the R package adegenet-2.1.1 (Jombart, 2008; Jombart and Ahmed, 2011). The data was filtered using the function 'filterdata' of the R package SambaR, allowing: 50% of missing individuals, 10% of SNPs missing data and distance of 500 bp between SNPs.

3.2 Structure analyses

Population structure has been investigated at different levels and using different methods with SambaR package. Correspondence analyses (CA) were performed using the function 'dudi.coa' of the R package ade4-1.7.13 (Dray and Dufour, 2007; Bougeard and Dray, 2018). Principal coordinate analyses (PCoA) were performed using the function 'pcoa' of the R package ape-5.3 (Paradis and Schliep, 2018) on distance matrices containing 2 different measures of genetic distance: Nei's genetic distance, calculated with the function 'stamppNeisD' of the R package StAMPP-1.5.1 (Pembleton *et al.*, 2013); Hamming's genetic distance, calculated with the function 'bitwise.dist' of the R package poppr-2.8.3 (Kamvar *et al.*, 2014). Geographical maps were generated with the function 'getMap' of the R package rworldmap-1.3.6 (South, 2011). Discriminant analysis of principal components (DAPC) was generated from genotyping-by-sequencing (GBS) for population genetic analysis in R (from http://grunwaldlab.github.io/Population_Genetics_in_R/gbs_analysis.html). In the end, samples have been coloured according a population assignment.

3.3 Migration rates

BayesAss3-SNPs was used to investigate recent dispersal between populations and hence recent gene-flow patterns (Mussmann *et al.*, 2019).
The number of iterations was set to 1000000, burn-in to 100000 and delta values to 0.1. Output matrices were converted into gene flow plots with the function 'plotmigration' of the R package SAMBAR, with use of the R package CIRCLIZE-0.4.6.

3.4 RDA

Association between population genetic structure and environmental parameters was assessed via a redundancy analysis (RDA) (Legendre & Legendre 2012). Mean annual values of sea surface temperature (SST), salinity (SAL) and Chlorophyll-A (Chl-A) have been extrapolated from each sampling area (downloaded from https://marine.copernicus.eu/). An analysis of variance

(ANOVA; 1000 permutations) was then performed to assess the global significance of the RDA and a marginal ANOVA (1000 permutations) was also run to determine if environmental PC factors were significantly correlated with allele frequencies.

3.5 Admixture analyses

ADMIXTURE is a program for estimating ancestry in a model-based manner from large autosomal SNP genotype datasets. To use ADMIXTURE, it's also needed to choose a K value for the number of ancestral populations. Ancestry coefficients were calculated with the software Admixture-1.3 (Alexander *et al.*, 2009) and plotted using SambaR. The output file for each parameter set are a Q-file (the ancestry fractions) and a P-file (the allele frequencies of the inferred ancestral populations). Four independent runs have been done for each value of *K* ranging from 1 to 4. The most likely value for *K* was given by the software analysis, exhibiting a low cross-validation error compared to other K values, reported in the output.

3.6 Molecular diversity indices

Differentiation between putative populations was assessed by estimating F-statistics in ARLEQUIN V. 3.5 (Excoffier & Lischer, 2010). Significance levels were determined through 10000 permutations. Number of usable loci for distance computation was 12029 (allowed level of missing data: 0.05). For each population, the observed and expected heterozygosity were calculated using ARLEQUIN V. 3.5 (Excoffier & Lischer, 2010).

3.7 Kinship

Kinship analyses was performed using the software PLINK, (Purcell *et al.* 2007). Estimates of identity by descent (IBD) proportion (PI_HAT) was calculated to find pairs of individuals who are genetically more similar than expected by chance in a random sample. PLINK '--genome' estimates relatedness of all pairs of samples and reports identify by decent (IBD, a

measure of whether identical regions of two genomes were inherited from the same ancestry). Unrelated individuals or are sample duplicate or MZ twins have PI_HAT close to 1; 1st degree relatives (parents/offspring or full-sibling) have PI_HAT close to 0.5; 2nd degree relatives (half-sibling) have PI_HAT close to 0.25; 3rd degree relatives (1st cousin) have PI_HAT close to 0.125; unrelated samples have PI_HAT close to zero. Secondary, GCTA SOFTWARE (Genome-wide Complex Trait Analysis) was used for calculating genetic relationships matrix (GRM) among individuals and populations (Yang *et al.*, 2011) and look for close kin groups.

3.8 Population Demography

The demographic histories of our populations were inferred using the Stairway plot analysis (Liu and Fu, 2015). Site frequency spectrum (SFS) for each analysed population has been calculated with ANGSD SOFTWARE (Korneliussen *et al.*, 2014). The stairway plot is a method for inferring detailed population demographic history using the SFS from DNA sequence data. It does not need a pre-defined population model and can be applied to hundreds of unphased sequences. Given the marked genetic structure and significant genetic differentiation found in the studied population, we ran stairway plot analyses for each sampled population individually. We set the generation time to 32 years and the mutation rate per site per generation to 2×10^{-8}, as initially proposed in Taylor *et al.* (2007) and performing 200 bootstrap replicates to estimate 95% confidence intervals.

4 Results

From initial number of 240 samples, a total of 160 samples comprised of biopsies from freeranging individuals (N=103), skin/muscle samples from stranded individuals (N=37) and sloughed skin (N=2) gave DNA quantity (500 ng) and quality (high molecular weight) suitable for dd-RADseq methodology. Samples have been pooled in 2 sequencing lanes, 80 samples each.

4.1 SNP calling and filtering

Both sequencing lanes combined produced 628.9 million reads. Reads were trimmed to 110 bp and demultiplexed and filtered using STACKS2.2 (Catchen *et al.*, 2013). Up to 16.2 million reads had to be discarded due to either low quality, an ambiguous radtag, or a missing read mate, resulting in an average number of 1.9 million read pairs per sample (stdev: 1.3 million, min: 0.003 million, max: 6.5 million). Individuals with less than 10. 1, 0.5 and 0.25 percent missing data had a minimum cover of respectively 3, 8.6, 8.6, and 23.2 reads. The mean sequence depth per individual per SNP ranged from 3.2 to 184.4 reads per SNP, with a mean and median of respectively 12.4 and 12.4 reads. After filtering 142 out of 160 individuals (38-66 per population) were retained (Fig. 4.1.1). After filtering 20759 out of 46717 SNPs were retained. Thinning reduced the data set further to 11537 SNPs. This filtered and thinned dataset was used for further analyses. The GC-content of the retained dataset equalled 0.61 and the 'transversion vs transition'-ratio equalled 0.62 (see Tab 4.1.1, SambaR summary statistic). The distribution of minor alleles, before filtering, after filtering and after thinning is shown in fig. 4.1.2.

	Before filtering	After filtering	After thinning
Number of individuals	160	142	142
Number of SNPs	46717	20759	11537
Percentage of SNPs with maf >= 0.05	20.81	24.83	25.36
Mean spacing between SNPs	51737	51152	93594
Median spacing between SNPs	4244	5964	53436
Mean proportion of missing data per individual	0.17	0.08	0.08
GC content	0.56	0.62	0.61
Transition vs transversion ratio	1.26	1.58	1.64

Table 4.1.1: SNPs dataset summary statistic from SambaR.

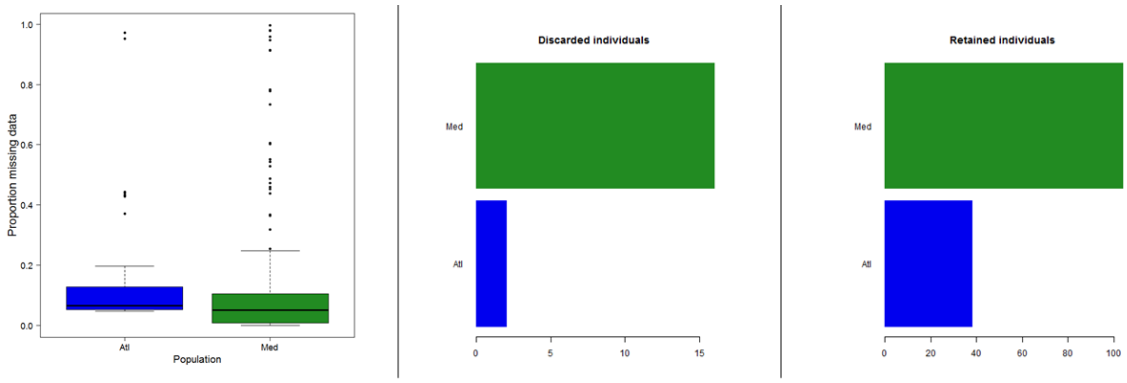


Figure 4.1.1: Proportion of missing data, number of discarded and retained individuals per Atlantic (At) and Mediterranean (Med) groups.

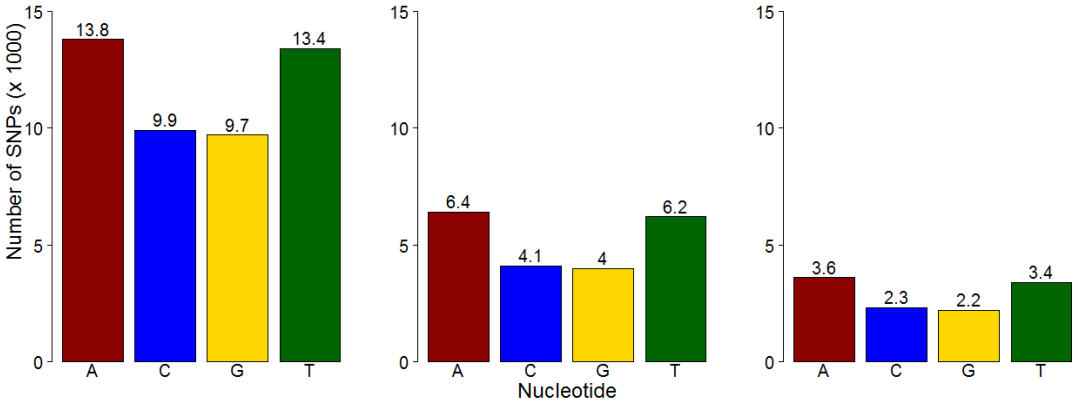


Figure 4.1.2: distribution of minor alleles before filtering, after filtering and after thinning

4.2 Population structure

The final genetic dataset consisted of 142 samples genotyped at 11537 biallelic SNP sites has been used to assess population structure. Initially, samples have been analysed considering sampling areas groups, in order to identify clusters between and within Eastern Atlantic and Mediterranean Sea. Ten different sampling areas have been selected: Azores, Canarias, Madeira, North Spain, Gibraltar Strait (Spain), Balearic, Ligurian, Tyrrenian, Adriatic and Greece in longitudinal order from West to East. Population clusters and sampling locations, shown in fig. 4.2.1, verified the existence of three distinct clusters: one including Atlantic samples and two different ones in Mediterranean Sea (Mediterranean A and Mediterranean B).

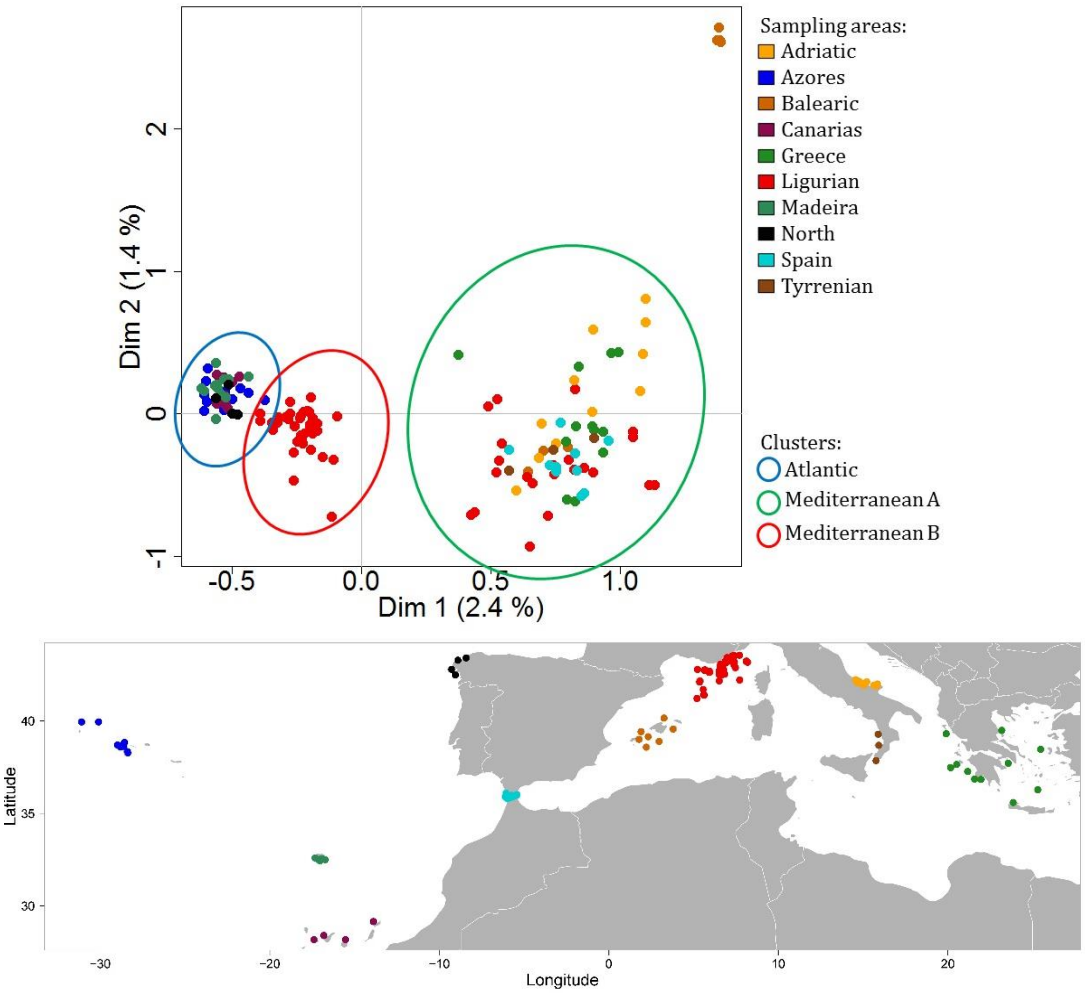


Figure 4.2.1: correspondence analyses with samples and clusters (on the top) and geographical map with sampling locations (on the bottom).

In detail:

- the Atlantic cluster (hereafter called "**Atl**") composed of 38 individuals sampled in Azores, Canarias, Madeira and North Spain areas from both stranding and skin biopsies events.
- A first Mediterranean cluster (hereafter called "**MedA**") composed of 66 individuals sampled in Gibraltar straits, Balearic Islands and Ligurian Sea from skin biopsies, and in Tyrrenian, Adriatic and Greek Sea from stranding events and 3 sloughed skin.
- A second Mediterranean cluster (hereafter called "**MedB**") composed of 38 individuals sampled in Ligurian Sea from freeranging individuals.

This latter is a key and unexpected result of this project. Considering these outputs, the vector file with putative populations has been reassessed as described. Samples have been categorized according to the above described clusters and coloured as follow: the blue dots represent Atl cluster, the red ones represent the MedB cluster and the green represent the MedA cluster (fig. 4.2.2 and 4.2.3). Nei's genetic distance between samples is showed in fig. 4.2.2 (on the right), with higher distance values (darker) identifiable among Atlantic and Mediterranean clusters and lower distance values (paler) between the two Mediterranean clusters.

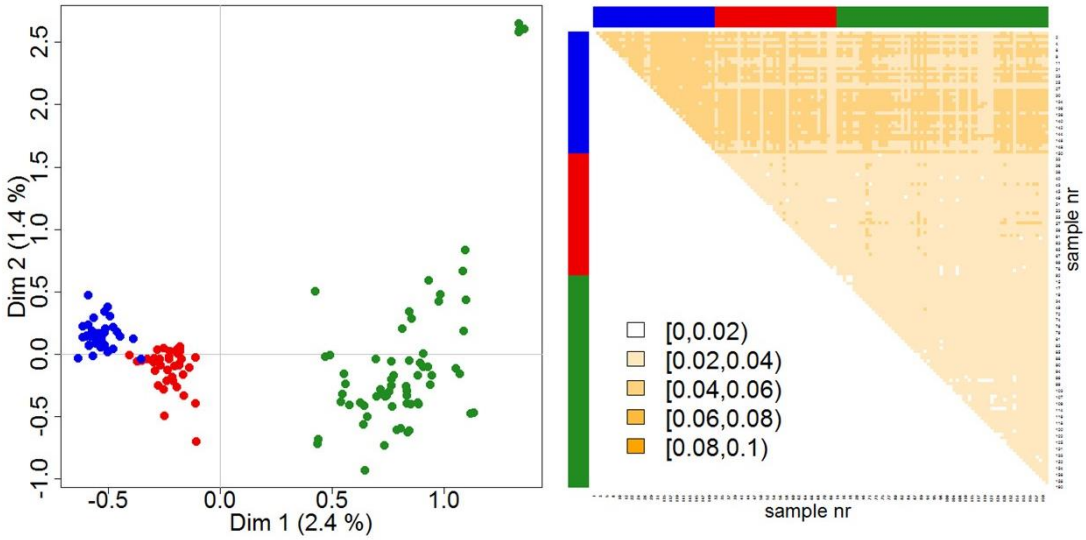


Figure 4.2.2: correspondence analyses plot and Nei's genetic distance. Blue = Atl; Green = MedA; Red = MedB.

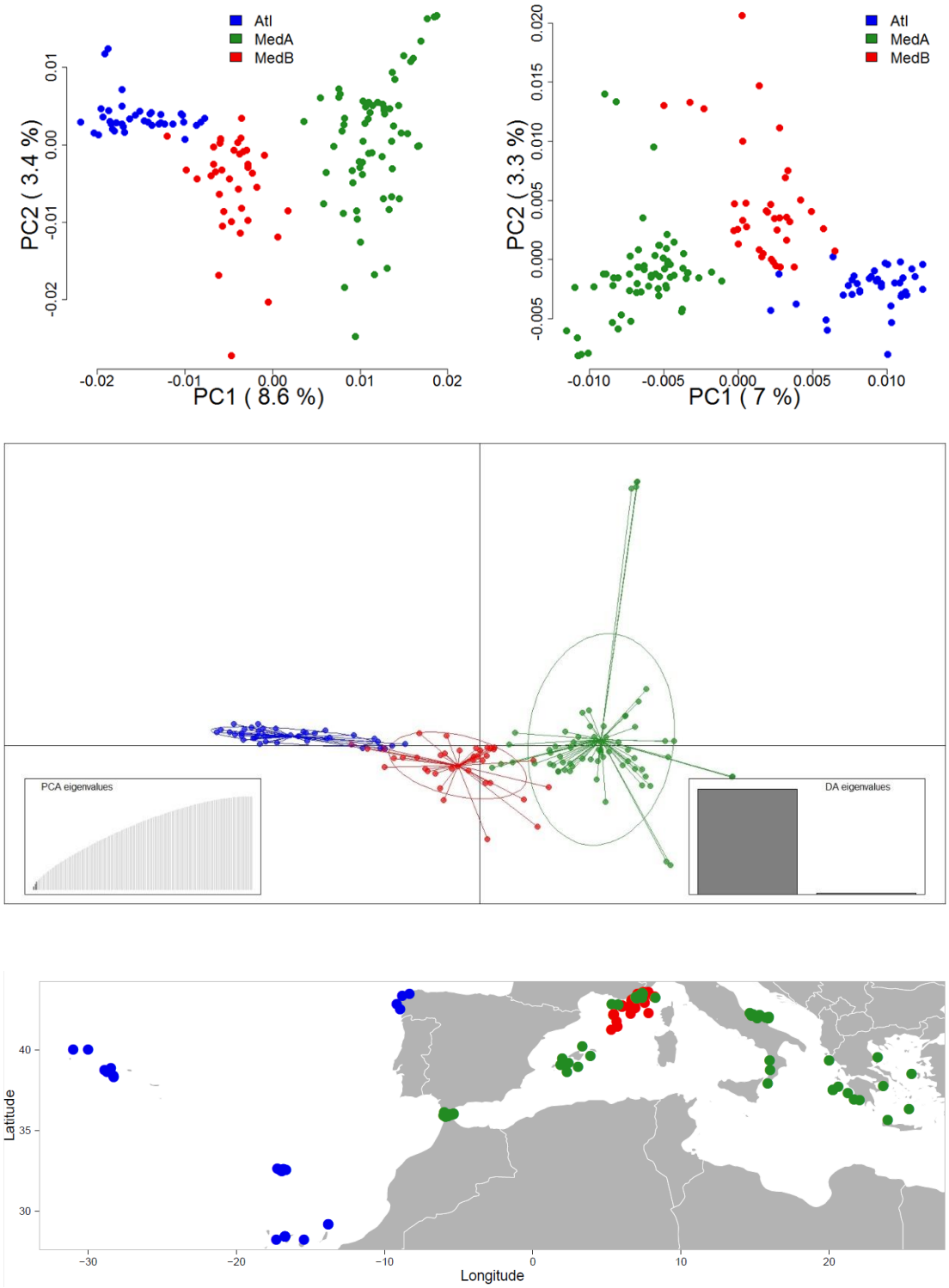


Figure 4.2.3: Principal coordinates analysis PCoA based on Hamming and Nei's genetic distance (in the top), discriminant analysis of principal components DAPC analysis plot (in the middle), map of sampling and cluster geographical locations with new colours assignment (in the bottom).
Blue = Atl; Green = MedA; Red = MedB.

To test the hypothesis of east west division within Mediterranean Sea, samples have been sorted in these 3 putative populations: Atlantic, West and East.

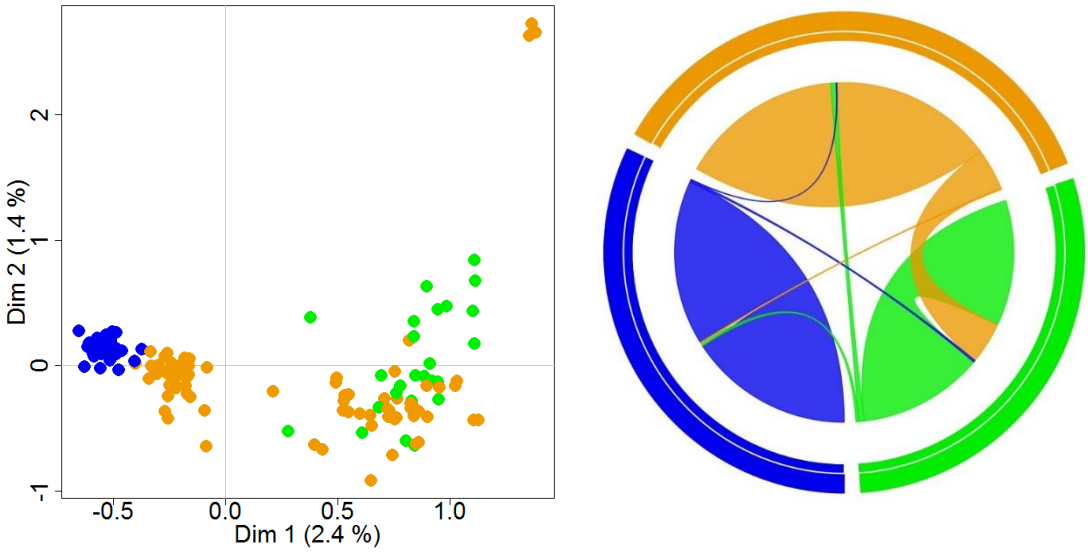


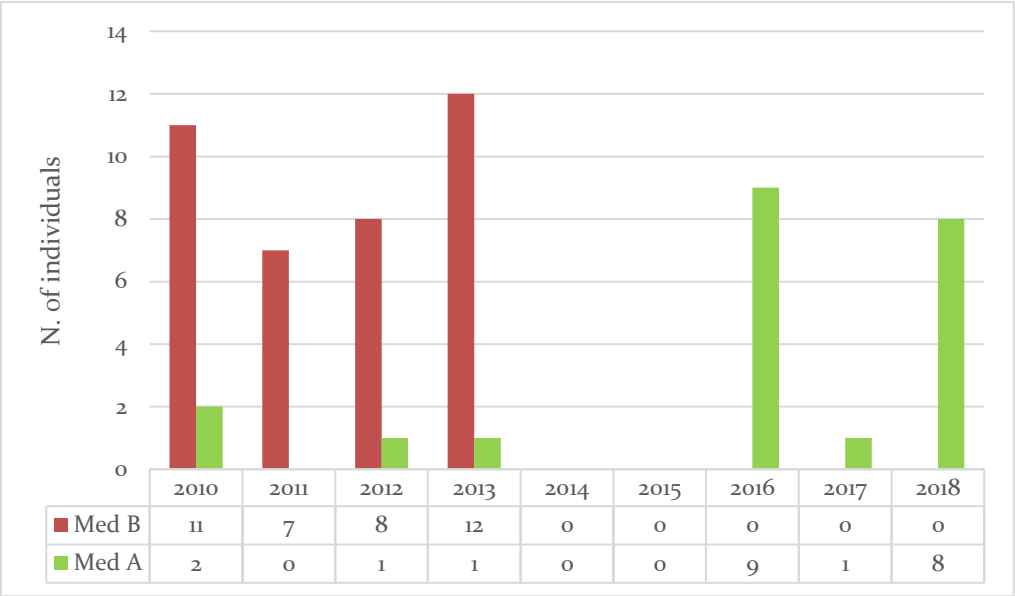
Figure 4.2.4: correspondence analyses plot (on the left) and Circocs plot migration rates (on the right) between the three populations.

Migration curve widths are proportional to the number of migrants.
Blue = Atl; Orange = West Med; Green = East Med

In the CA plot (fig 4.2.4 on the left) some samples in orange dots, representing individuals sampled in the western basin, overlap with individuals sampled in Eastern basin, showed in green. No division has been found also in the migration rate plot (fig 2.4.2 on the right): the yellow curve reflects the gene flow towards the eastern basin. Migration curve widths are proportional to the number of migrants between putative populations.

4.3 Analyses of MedA and MedB occurrence in Ligurian Sea

Considering the sampling area of Ligurian Sea, two groups genetically separated have been found: one corresponding to MedB cluster and a second one composed of 18 samples which are found within MedA. Considering sampling years, all individuals sampled in this area, have been collected in summer months (from June to September) since 2010 to 2018. However, analysing MedA and MedB occurrence in Ligurian Sea, samples belonging to MedB population have been collected between 2010 and 2013. Differently, samples of MedA population have been collected between 2016 and 2018, except 4 of 22 ones collected in 2010-2013 years (fig. 4.3.1).



	2010	2011	2012	2013	2014	2015	2016	2017	2018
Med B	11	7	8	12	0	0	0	0	0
Med A	2	0	1	1	0	0	9	1	8

Figure 4.3.1:. Occurrence of MedA and MedB populations in Ligurian Sea

4.4 Sex ratio

Gender determination was determined genetically for all 103 samples collected from free ranging individuals (Fig. 4.4.1; see Tab. S2 in Appendix for details). Sex of 39 samples from stranded individuals has been assessed during necropsy. The sex ratio of males to females was calculated for each population and compared with an expected ratio of 1:1. For Atl population, marginally insignificant male to female ratio was of 0.41:1 (χ^2=3.525, *P*=0.06). For

Mediterranean population, significant male to female ratio emerged: 5.33:1 for MedB (χ^2= 10.07, *P*<0.005) and 2.67:1 for MedA (χ^2= 7.190. *P*<0.05).

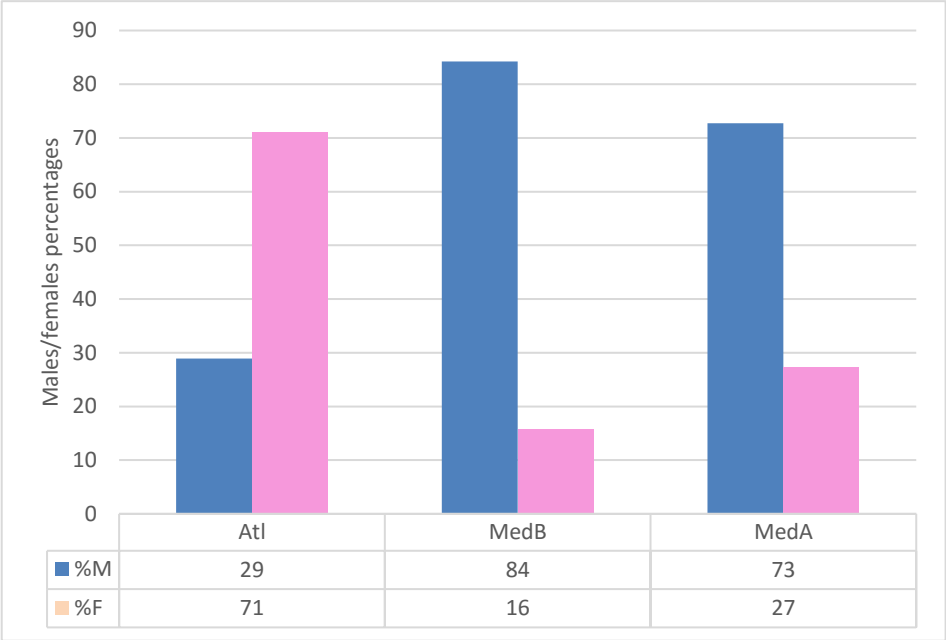


Figure 4.4.1: histogram of males and females percentages distribution between identified population. Males in blue, females in pink.

4.5 Molecular diversity

Pairwise difference using *Fst* has been calculated with ARLEQUIN 3.5 SOFTWARE. This analysis has been performed considering initially the Atl Vs. the whole Mediterranean dataset, revealing a significant *Fst = 0.03454 (P-value=0.000)*. Secondarily, pairwise comparison has been conducted with the identified Atl, MedA and MedB populations (see tab 4.5.1).

	Atl (n=38)	**MedB (n=38)**	**MedA (n=66)**
Atl	-		
MedB	0.02512 (P=0.00±0.00)	-	
MedA	0.03960 (P=0.00±0.00)	0.01037 (P=0.00±0.00)	-

Table 4.5.1: Pairwise difference using Fst (with P-values in parenthesis) between identified population. N is the number of samples to each population.

Higher genetic differentiation has been found for Atl/MedA and Atl/MedB comparisons, respectively, 0.03960 and 0.02512. Lower difference

has been detected among two Mediterranean clusters, 0.01037. All comparisons are highly significant (*P-value*=0.000). Another test has been run considering three geographic strata: Atlantic, Ligurian (all samples collected in Ligurian) and the left Mediterranean (all med samples except Ligurian ones). All pairwise comparisons have shown significant results and support the differentiation of the Ligurian stratum from the other Mediterranean stratum (tab. 4.5.2).

	Atl (n=38)	**Lig (n=60)**	**Med (n=44)**
Atl	-		
Lig	0.03153 (P=0.00±0.00)	-	
Med	0.03981 (P=0.00±0.00)	0.01400 (P=0.00±0.00)	-

Table 4.5.2: Pairwise difference using Fst (with P-values in parenthesis) performed between samples collected in Atlantic (Atl), Ligurian (Lig) and all the left Mediterranean (Med).

4.6 Migration Rates

Recent gene flow rates were inferred for the 11537 SNPs dataset in BayesAss3-SNPs. The estimates are visualized as a Circos plot in fig. 4.6.1 and displayed in tab. 4.6.1. The three identified populations are coloured as described before (Atl in blue, MedB in red and MedA in green). From Circos plot, migration curve widths are proportional to the number of migrants among identified populations. The posterior estimates suggest a general trend of low geneflow rate coming from Atlantic Ocean towards Mediterranean (respectively 3% toward MedB and from 0.4% to null toward MedA) (showed in tab 4.6.1). These values reveal a stronger link with MedB than MedA. From Mediterranean to Atlantic, values range from 0.8% to null and confirm the absence of outward geneflow. Among Mediterranean clusters, geneflow appears to be minimal, with values from 2% to 1% from MedB to MedA and 1% from MedA to MedB.

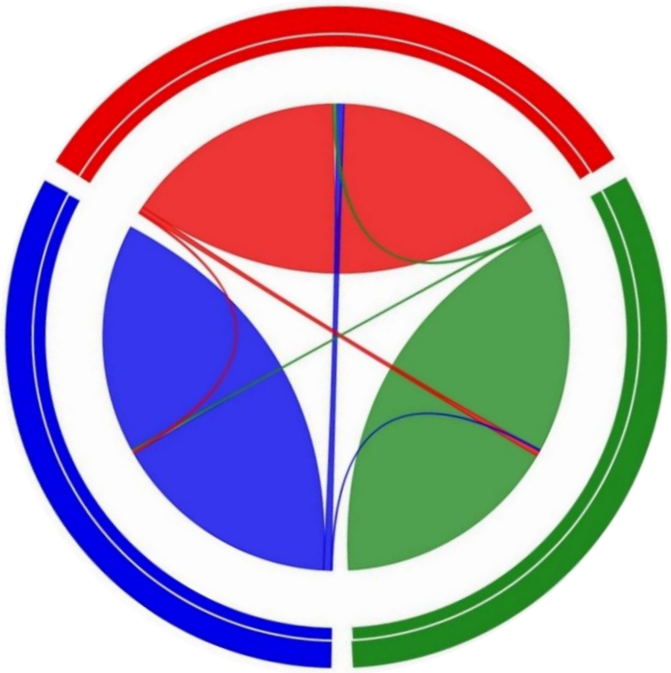


Figure 4.6.1: Migration rates between the three populations. Migration curve widths are proportional to the number of migrants. Atl = blue; MedB = red; MedA = green.

Source / Current	Atlantic	Mediterranean A	Mediterranean B
Atlantic	0.9836(0.0113)	0.0084(0.0083)	0.0080(0.0079)
Mediterranean A	0.0048(0.0047)	0.9724(0.0111)	0.0227(0.0103)
Mediterranean B	0.0325(0.0153)	0.0145(0.0278)	0.9529(0.0315)

Table 4.6.1: Posterior mean estimates for migration rates. Rates defined as the proportion of individuals in a 'Current' population that are migrants derived from a 'Source' population. Standard deviations given in parentheses.

4.7 Admixture

Ancestry proportion of each individual and population have been estimated and visualized using ADMIXTURE 1.3 SOFTWARE (Alexander *et al.*, 2009). Analyses have been performed with different K values. The less CV error scores have been for k=2 and k=3 respectively 0.1755 and 0.1797. K=1 and K=4 have given higher errors values (K=1: 0.1808; K=4: 0.2088). The admixture bar plot and map are shown in fig 3.6.1 for k=2 and in gig 3.6.2 for k=3. Both k=3 and K=2 revealed admixture in the MedB cluster. Atlantic ancestry is shown in blue. Mediterranean ancestry is shown in green for k=2 (fig. 4.7.1-2) and in green and orange for k=3 (fig. 4.7.3-4). Within MedA cluster, analyses with k=3 reveals two *subclusters* coloured in orange and green – despite they are not separate in the PCoA and CA. In details:

- oranges samples are mostly from Ligurian Sea and Gibraltar straits, respectively 19 and 10 individuals, plus 3 individuals from Balearic Islands, 2 individuals from Tyrrenian sea, 3 individuals from Adriatic and 2 individuals from Greece.
- greens samples are mostly from Greece (n=11) and Adriatic Sea, respectively 11 and 8 individuals, plus 3 individuals from Ligurian Sea, 3 individuals from Balearic Islands, and 1 individual from Messinian Straits.

The central section of the ancestry plot deserves the main importance of these analyses. Indeed, the second Mediterranean cluster (MedB) – identified by red dots in previous CA, PCoA, DAPC and Circos Plots (see fig. 4.2.2) is characterized by admixture between Atlantic and MedA cluster (respectively blue-green for K = 2, blue-green-orange for k = 3). Sperm whales within this latter cluster have been sampled in Ligurian Sea between 2010 and 2013 as shown in fig. 4.3.1.

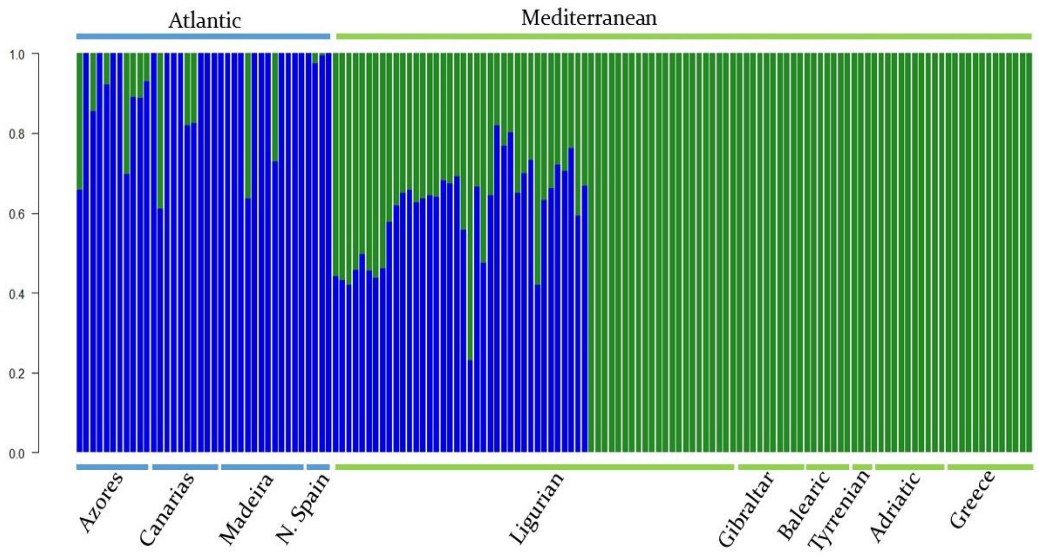


Figure 4.7.1: Admixture analyses for ancestry estimation K=2. Each individual is represented by a vertical bar broken into different coloured genetic clusters, with length proportional to probability of assignment to Atlantic (in blue) or Mediterranean (in green) ancestries. Sampling locations groups are listed in the bottom.

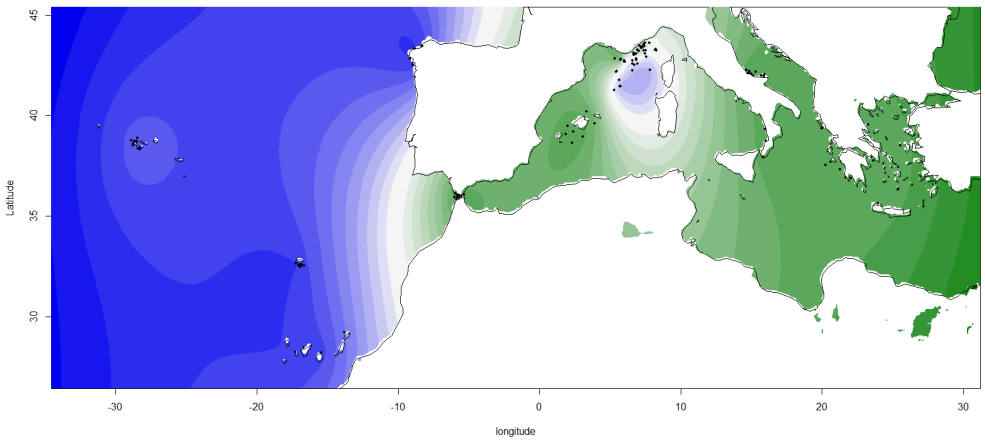


Figure 4.7.2: Admixture results plotted on geographical map. Atlantic ancestry in blue and Mediterranean ancestry in green. Darker and paler colours reflect higher and lower ancestry score respectively. In black dots are the sampling locations.

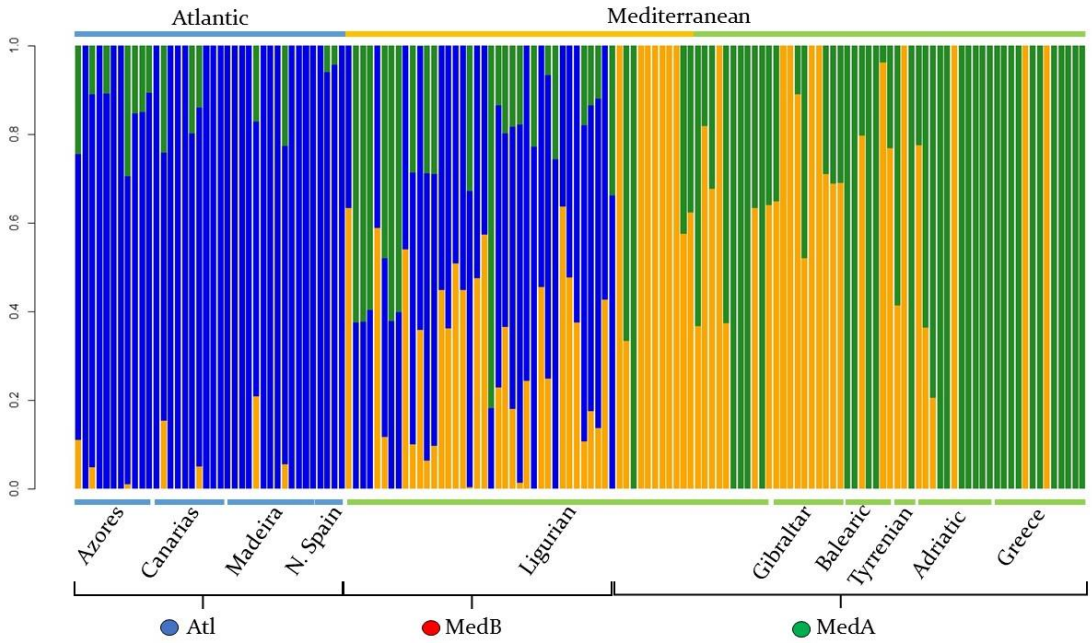


Figure 4.7.3: Admixture analyses for ancestry estimation K=3.
Each individual is represented by a vertical bar broken into different coloured genetic clusters, with length proportional to probability of assignment to Atlantic (in blue) or Mediterranean (in orange and green) ancestries.
Ten sampling locations and the three identified clusters (with blue, red and green dots, as shown in fig. 4.2.2 and 4.2.3) are listed in the bottom.

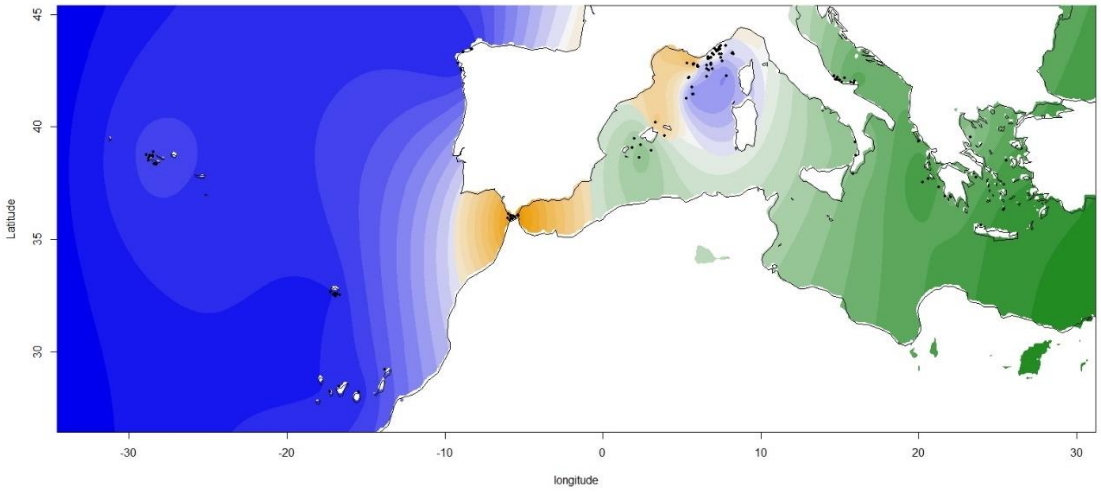


Figure 4.7.4: Admixture results plotted on geographical map.
Atlantic ancestry in blue and Mediterranean ancestries in orange and green.
Darker and paler colours reflect higher and lower ancestry score respectively.
In black dots are the sampling locations.

4.8 Redundancy analysis

Redundancy analysis (RDA) method was selected for the analysis of populations-environment relationship using three environmental factors, sea surface temperature (SST), salinity (SAL) and chlorophyll-a (ChlA) as possible explanatory variables. RDA was performed on the 5938 SNPs potentially under divergence (0.05 significance level) as the response variables. The RDA axes 1 and 2 represented 59.0 % 25% respectively. The marginal ANOVA showed that PC factors 1 (SST), and 2 (SAL) were significant predictors of the populations structure with *P*-values < 0.001 (respective F= 3.1018, F= 1.3169). Chl-A wasn't significative with *P-value=0.954* (fig. 4.8.1).

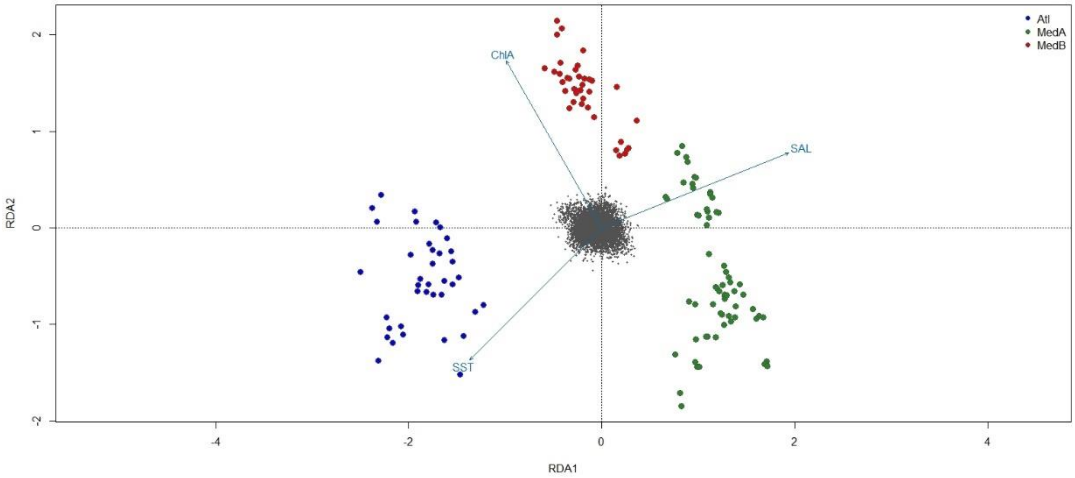


Figure 4.8.1: Redundancy analysis
Axis 1 (59.0% of variance) and 2 (25.0% of variance) showing population structure for the 5938 SNPs markers potentially under divergence at the 0.05 significance level (plus marks) and related environmental PC factors, sea surface temperature (SST), salinity (SAL) and chlorophyll-a (ChlA) as blue arrows. Populations colour legend on the top right.

4.9 Kinship

Kinship analyses has been assessed calculating identity by descent (IBD) proportion (pi_hat value) within and among populations (fig. 4.9.1). Within populations mean of pi_hat values are lower in Atlantic (0.000±0.014) than Mediterranean A (0.097±0.153) and Mediterranean B (0.087±0.124) population (see tab S4-5-6 in appendix for details). Among populations, mean of pi_hat pairwise is low for Atl vs. Med clusters (Atl vs. MedA = 0.005±0.029; Atl vs. MedB = 0.012±0.040) and higher between Med clusters (MedA vs. MedB 0.073± 0.115). These estimates are visualized in violin plot in Fig 4.9.1: the higher proportion of individuals both within and among population are in the range from 2nd degree relatives (pi_hat≈0.125) to unrelated (pi_hat=0). Similar trend is in fig 4.9.2, that shows the proportion of IBD per individual.

Consistent to pi_hat pattern, relatedness values calculated with GCTA SOFTWARE are illustrated in heatmap plots. Genetic relatedness matrix has been assessed within (fig. 4.9.3) and among population (fig. 4.9.4). The general trend within and among population is of unrelatedness. Some low levels of kin are found within Mediterranean Sea. In Ligurian Sea, sampling events have been performed on different groups in different years but also on different individuals within the same group. In this latter case, weak levels of relatedness (between second and third degree, from 0.25 to 0.125) have been found both in MedB population and in Ligurian group within MedA population (Fig. 4.9.3). In the same matrix, a similar trend of weak relatedness levels is shown within each sampled area, and between Adriatic and Greek areas. However, in all comparisons no close kin groups have been found.

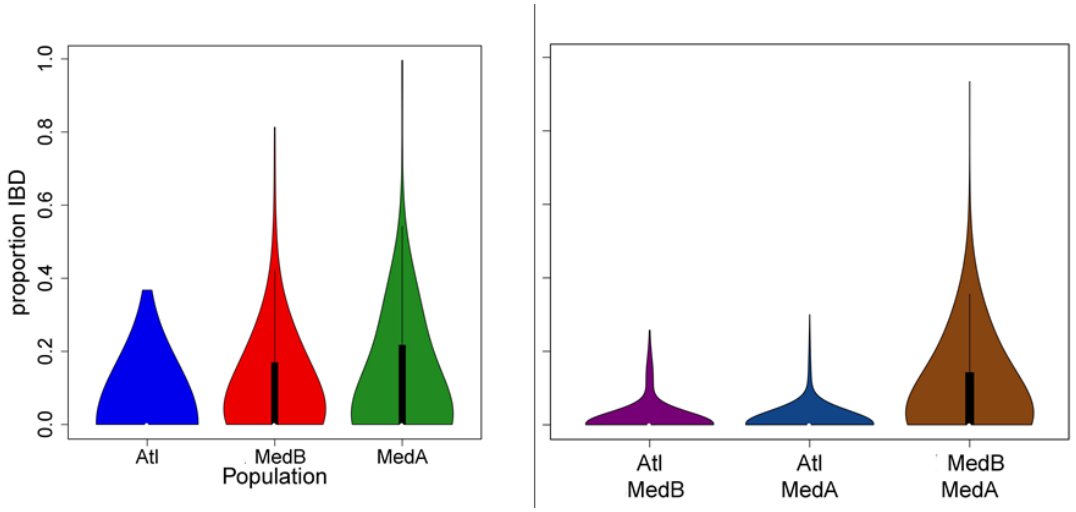


Figure 4.9.1: violin plot of pi_hat value calculated within each population (on the left) and among population pairwise (on the right).

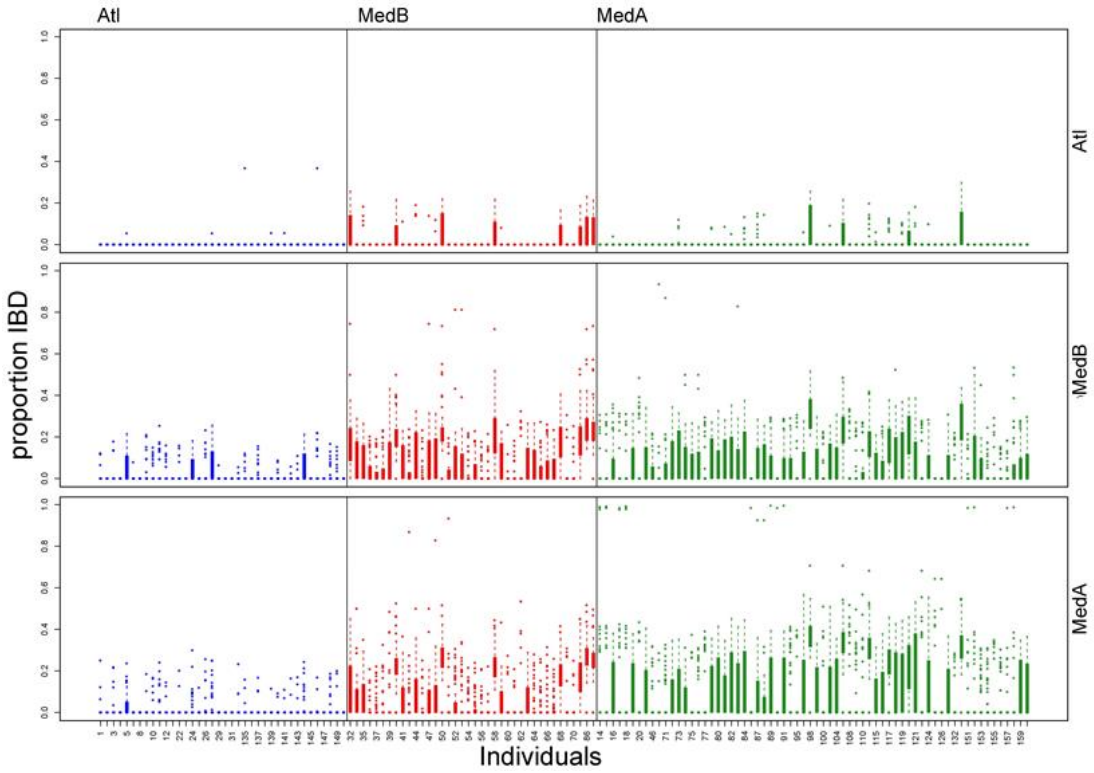


Figure 4.9.2: bar plots of pi_hat values per individual, among and within populations.

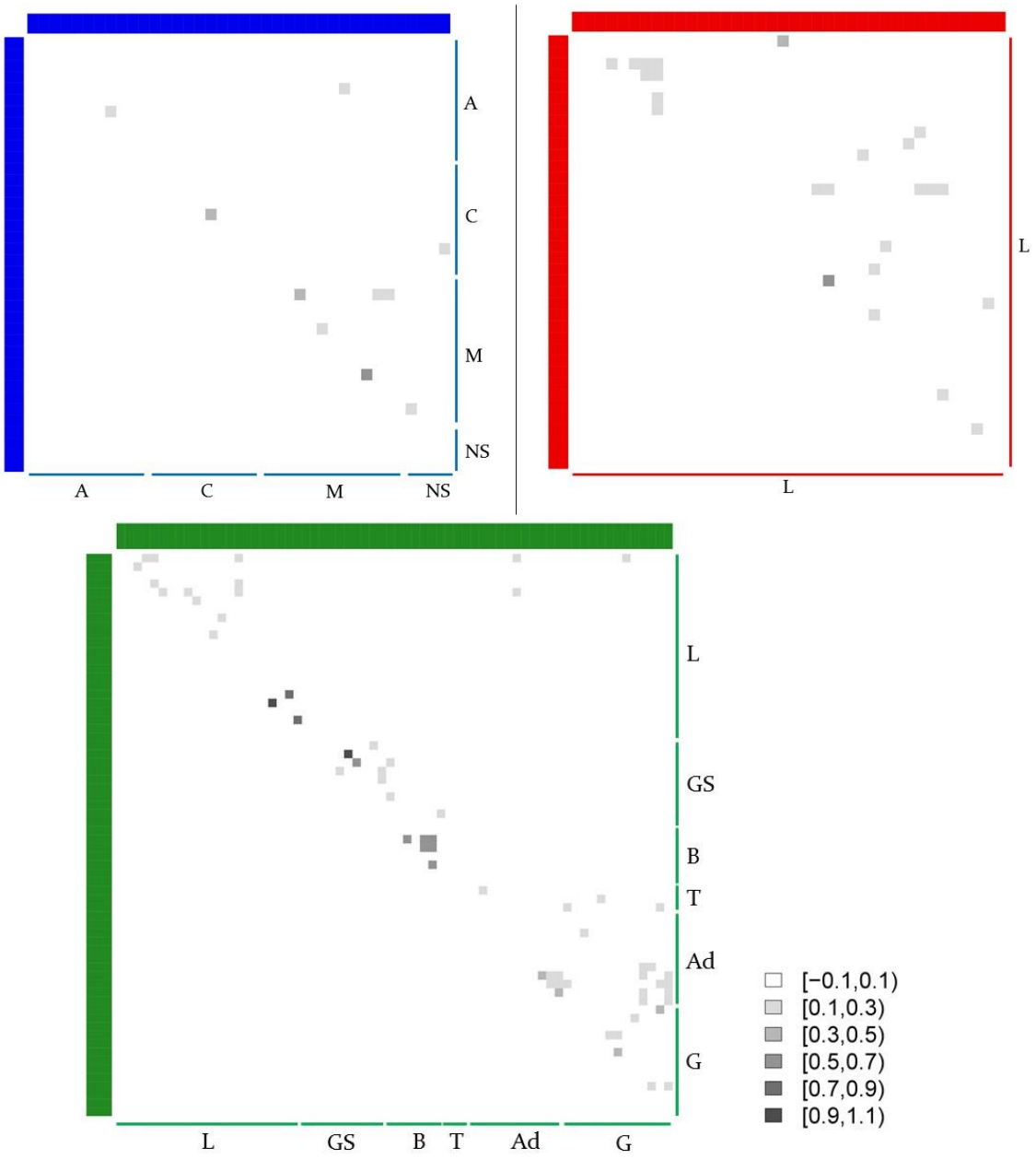


Figure 4.9.3: Genetic relatedness matrix for each population. Atlantic population in blue, with 38 individuals. MedB population in red, with 38 individuals. MedA population in green, with 66 individuals. Relatedness scale in the bottom right: higher values identified by darker colours correspond to closer related couples. Sampling areas abbreviations on the axis: A=Azores; C=Canarias; M=Madeira; NS=North of Spain; L=Ligurian Sea; GS=Gibraltar Straits; B=Balearic Islands; T=Tyrrenian Sea; Ad=Adriatic Sea; G=Greek Sea

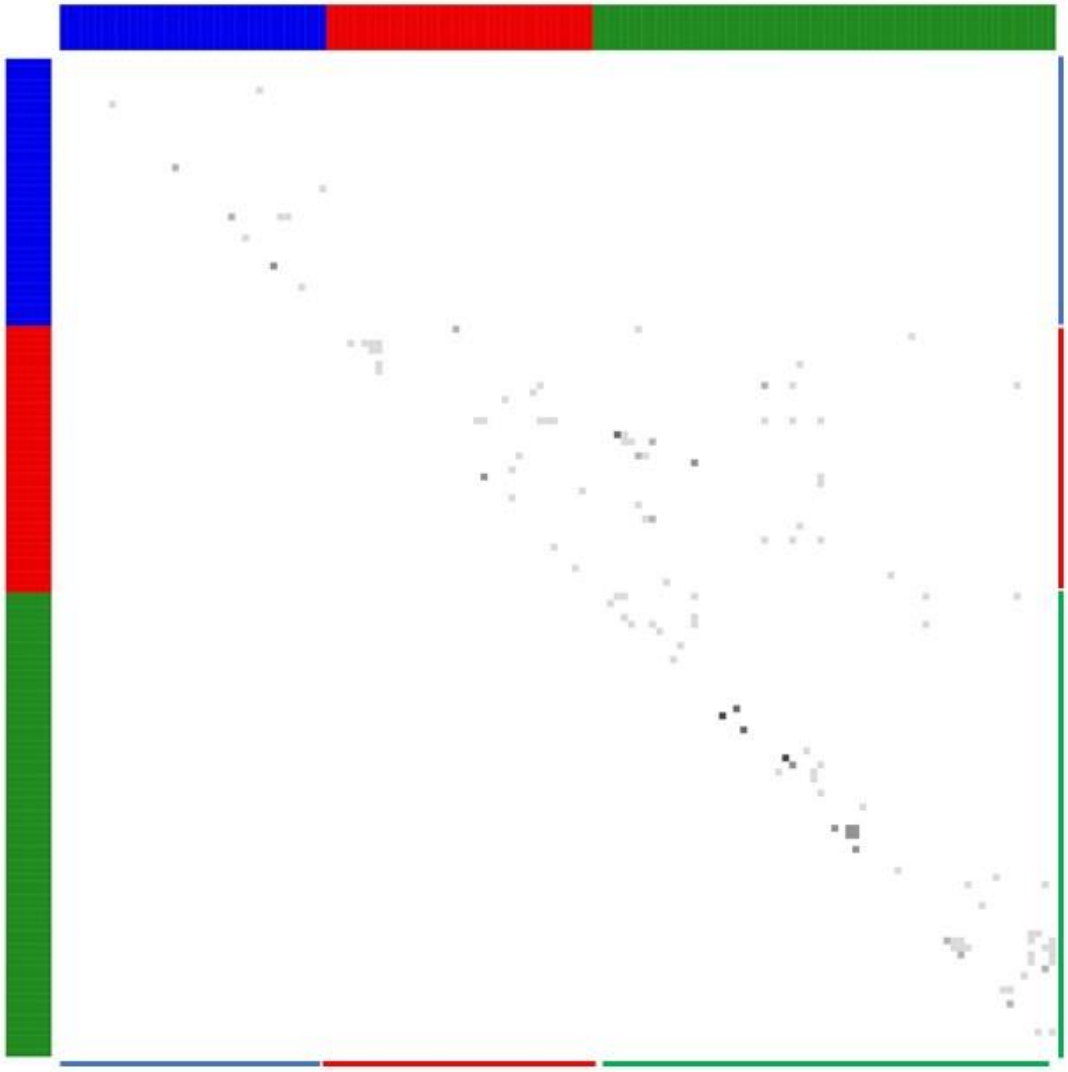


Figure 4.9.4: Genetic relatedness matrix among populations.
Atlantic in blue; MedB in red; MedA in green.
Relatedness scale as shown in fig. 4.9.3.

4.10 Demography

The demography of Atlantic and Mediterranean populations was inferred using the Stairwayplot analysis (Liu and Fu, 2015). We used an estimate of generation time of 32 years and 2 x 10^{-8} as mutation rate (see Taylor *et al.* 2007). For Atl population, the trend identified a strong population bottleneck event, between 20k and 100k years ago, corresponding to the last glaciation maximum. A second and recent reduction is probably reflecting whaling activities (fig 4.10.1).

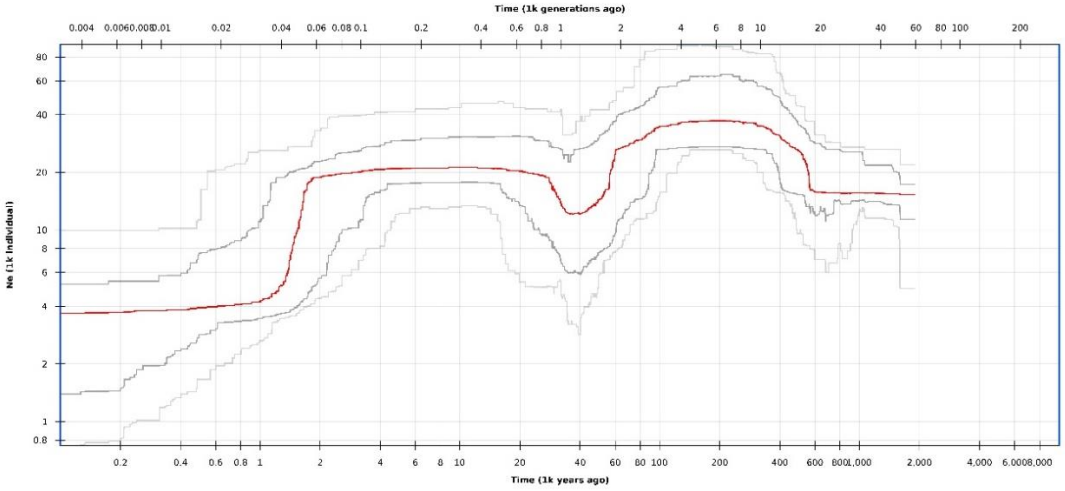


Figure 4.10.1: Atlantic population demography inferred through stairway plot. Red line indicates median values

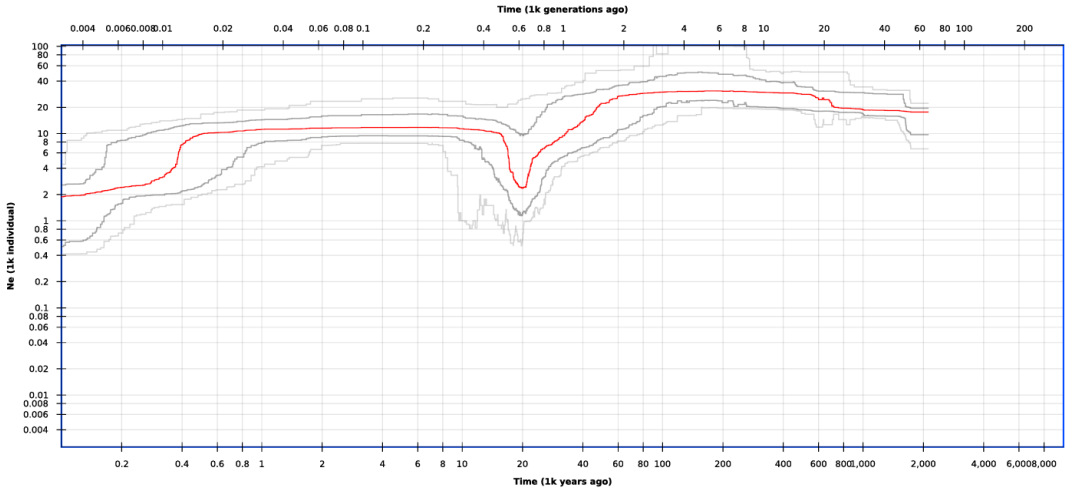


Figure 4.10.2: Mediterranean A population demography inferred through stairway plot. Red line indicates median values

As for Atlantic population, in Mediterranean Sea populations a similar trend has been found, with a marked bottleneck event corresponding to last glacial maximum and a reduction in the last centuries (fig. 4.10.2). However, the Ne scale for Mediterranean populations has lower values and reveals an actual smaller effective population size comparing with Atlantic. In general, MedA trend seems to reflect Atlantic one. Differently, the analyses on MedB population reveals a general reduction, but probably considering the admixture of this cluster, the used model is not suitable for inferring population size in this case (see MedB in fig 4.10.3).

Analysing the general trend of the three plots in fig. 4.10.3, summarily we can address: a bottleneck event corresponding to LGM; a recent Ne reduction; Ne higher in Atlantic (Ne<10000) than Mediterranean (Ne<2500).

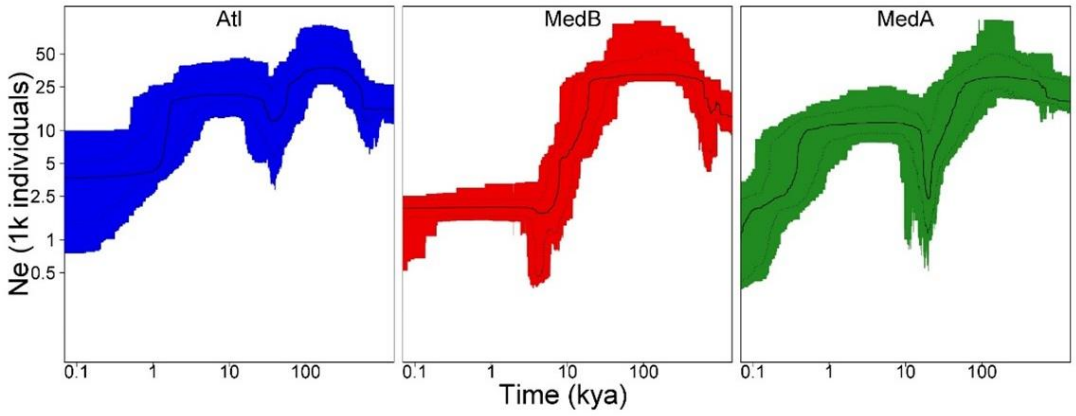


Figure 4.10.3: Stairway plots showing demographic histories of the identified population.
Mutation rate: 2 x 10^{-8} per site per generation, generation time: 32 years. Bold lines indicate median values, whereas dashed line indicate 12.5% and 87.5% percentile values. Colour margins indicate 2.5% and 97.5% percentile values.

'Moby Dick' by Matthew Greskiewicz

5 Discussion

This study is the first one that applies dd-RADseq methodology (Baird *et al.*, 2012) on sperm whale to detect a large number of SNPs. In Mesnick *et al.* (2011) 36 SNPs (plus mtDNA CR and 6 microsatellites loci) have been used to assess population structure in the North Pacific Ocean. No studies to date have addressed sperm whale population structure using SNPs, within Mediterranean population and its neighbour Atlantic one. ddRADseq is confirmed to be an efficient and cheap methodology, in order to obtain high number of SNPs (11537 after filtering and thinning quality controls). Considering that this analysis is not expensive and gives a huge number of SNPs, it should be promoted for population genetic studies on conservation genetic of species living in small and close area. Its only weakness is the need to have DNA with really good quality (high molecular weight) and quantity (about 500 ng). In this study, 90 samples have been discarded because of low DNA quality and quantity. These samples were mostly sloughed skin – collected after sperm whale fluking and therefore in a complete non-invasive way. Unfortunately, DNA in the last layers of the skin is in low concentration and really fragmented, thus not useful for the starting point of this methodology. Some other discarded samples were from stranded individuals, probably because of the advanced decomposition state of the dead animal. All skin biopsies samples collected from free-ranging individuals gave the best DNA, both for quality and quantity. Considering previous studies that used sperm whale samples from the Mediterranean Sea, this is the 3rd study. Furthermore, considering our high number of samples from the Mediterranean area and our objectives, focused **mainly** on the Mediterranean

population, this is the first study aiming to clarify population structure and dynamic and pattern of connectivity within this area.

5.1 Population structure

In assessing population structure, the results were consistent in all performed analyses: PCoA, PCA, DAPC, RDA, Ancestry, Gene flow analyses and measures based on summary statistics. Structure within Mediterranean and among Mediterranean and Atlantic will be discussed separately.

5.1.1 Atlantic Vs. Mediterranean

The expected differentiation among Atlantic and Mediterranean Sea described in previous studies was confirmed again by our SNPs marker. Through photo-identification and acoustics (see review of Rendell and Frantzis, 2016) and genetics (see Drouot et a., 2004; Engelhaupt *et al.* 2009), several studies supported well the hypothesis of a Mediterranean population separated from Atlantic population. Similarly, our PCoA, PCA, DAPC, and RDA analyses reveal a clear separation between Atlantic and Mediterranean clusters. Pairwise comparison between these two areas reveals a low but significant value (*Fst=0.03454, P-value=0.000*), consistent to Engelhaupt *et al.* (2009) values and results (*Fst=0.034, P-value=0.022*; see tab. 7 in Engelhaupt *et al.*, 2009). Significant genetic division between Atlantic and Mediterranean areas has already been documented for other cetaceans species, such as fin whale (Schleimer *et al.*, 2019), Cuvier's beaked whale (Dalebout *et al.*, 2005) and for other top predators such as blue fin tuna (*Thunnus thynnus*). However, Engelhaupt *et al.* (2009) revealed that females exhibit site-fidelity in the Mediterranean basin, with marked differentiation between Med and Atl at mtDNA, but lower divergence at microsatellites loci, suggesting a male biased gene flow. Unfortunately, our genetic dataset is based on SNPs from wide genome coverage and not only on sex chromosome (as can be done using microsatellites or mtDNA), and therefore it is not possible to detect sex

specific markers in order to have results on sex biased geneflow as Engelhaupt *et al.* (2009) did. However, our key point was to find evidence and confirm of **low** gene flow towards Mediterranean which could maintain genetic diversity and reduce possible inbreeding phenomena.

Indeed, estimates of contemporary migration patterns indicate asymmetric and eastwards gene flow, between these two areas (see fig 4.6.1 and tab 4.6.1). These results suggest that recent low migration rate between Atlantic and MedB exists, limited to 3% of migrants per generation, but enough to cause Atlantic "signal" in Mediterranean basin. This result reveals that some individuals move into the Mediterranean Sea, crossing Gibraltar straits and probably heading to the northern part of western basin. This is consistent with results of Engelhaupt *et al.* (2009) on males inward movements, as discussed before. Sperm whales have been routinely observed in Gibraltar, both in summer (de Stephanis *et al.*, 2008) and winter (Gauffier *et al.*, 2012). Historical reports from Bolognari (1949; 1950; 1951), gave evidence of seasonal movements through the straight - entering in the winter and going out in spring – and with inward sightings more than outward ones. The north west Mediterranean, thanks to a complex network of submarine canyons that act as corridors between continental shelf areas and the deep-sea regions, playing a major role enhancing oceanographic processes, enriching the deep-sea food web (De Leo *et al.* 2010) and creates a favourable environment to cephalopods (e.g. Quetglas *et al.*, 2000; O'Dor and Coelho, 1993), that are sperm whales preys. Therefore, this region can support the energetic demand of sperm whales and others deep divers. However, we can speculate that, what may drive Atlantic sperm whale to the "oceanic" regions of North West Mediterranean Sea is firstly the "exploring instinct of mammals" and secondarily the looking for both food resources and possibly females. Exploring is a form of animal behavior directly linked with cognitive and social functioning in mammals (Pisula 2008). Home ranges usually depends on the habitat quality, group size, behavioral strategies, competitors and/or predators and all these features are unique for every habitat and shape the species-

specific home range (Fryxell *et al.*,1988). Movements and migrations have evolved as a strategy to maximize fitness in order to face seasonal and spatial variation in resources (Boyce 1979, Swingland and Greenwood 1983). For sperm whales, analyses of stomach content revealed the *H. bonnellii* is the favourite prey both in Mediterranean Sea and in Atlantic Ocean (see Garibaldi and Podestà, 2014; Clarke et al, 1993). In Atlantic, sperm whale can also feed on squids of *Ommastrephidae* family, that are muscular squids, fast swimmer and with higher calorific power, suitable for big sperm whale with have high energetic demand. Ommastrephidae squids occur also in Mediterranean, as found in the stomach content of Cuvier's beaked whale (Garibaldi and Relini, 2005) and pilot whales, but they are found in low percentage within sperm whale stomach content (Garibaldi and Podestà, 2014). These findings exclude the chance that some sperm whale may enter in Mediterranean Sea because of "special dietary needs": but probably, considered the small population size in this area and less competition for food, and the high abundance of squids, Atlantic sperm whale have found a suitable environment along their movements. During these expeditions, probably done by only males, after finding suitable habitat for feeding, they focused on females as a resource. These latter focus on suitable environments for breeding and parturition (Greenwood 1980) and Mediterranean Sea has no predators to treats their offspring. Our admixture results (fig. 4.7.1-4) give evidence of breeding activity of some Atlantic individuals with Mediterranean sperm whale, giving birth to an hybrids lineage. However, the low but significant divergence and population structure confirm that these movements through Gibraltar Straits are not regular (sperm whale come and go repeatedly) but at the same time make Mediterranean population not completely isolated.

In this framework, it is really important to underline that contemporary migration patterns may not reflect historical ones. Restricted movement between both basins and the North Atlantic has been suggested for bluefin tuna (*Thunnus thynnus*; Boustany *et al.* 2008). Recent evidence on movements through Gibraltar have been found in other cetaceans species: fin whale

movements suggest a migration of a small community of fin whales through the Strait of Gibraltar, with remarkable seasonal directionality - towards the Atlantic Ocean between May and October, and towards the Mediterranean Sea between November and April (Gauffier *et al.*, 2018). Our results reveal that Strait of Gibraltar and the Almeria–Oran front are moderate to weak barrier to the gene flow dispersal of sperm whale, and not strict barrier as found for other species (i.e. Gaspari *et al.*, 2007; Guarniero *et al.*, 2002).

Up to now, it is well known that sperm whales from Mediterranean and Atlantic show differences in body size (Mediterranean ones are smaller, see body size par. in Ch. 1), in coda pattern (Mediterranean have "3+1" pattern as a main one, but not the only, see Acoustic review par. in Ch. 1) and in the fluke contour (Atlantic flukes are more marked than Mediterranean one, personal observation and Maurizio Würtz personal communication). Considering this last point, individuals with flukes contour similar to "Atlantic type" have been sighted in the Ligurian Sea. No such data have been published yet, but these observations supports an inwards movement from Atlantic.

These new findings on gene flow into the Mediterranean are important information for the future of Mediterranean sperm whale population. The low migration rates can probably have a positive role in order to avoid the loss of genetic diversity within Mediterranean Sea and reduce inbreeding phenomena.

5.1.2 Structure within the Mediterranean

Within the Mediterranean, the main hypothesis we tested was the division of the Mediterranean population in Easter and Western clusters, as suggested for other species (see bottle dolphin in Gaspari *et al.*, 2015; striped dolphin in Gaspari *et al.*, 2007). In analysing results from CA, PCoA, DAPC where Mediterranean population was divided in MedA and Medb (see fig. 4.2.1-3) cluster and analysing CA plot and Circos plot (see fig. 4.2.4) where Mediterranean population was divided in WestMed EastMed, no evidence of West-East separation has been found. Within MedA cluster are samples collected in all selected areas: Gibraltar Straits, Balearic Islands, Ligurian Sea, Tyrrenian Sea, Adriatic Sea, Hellenic Trench and Aegean Sea. In the fig. 4.2.2 samples from West overlap with samples from the East, too. These results strongly suggest an open connection between Western and Eastern basins of Mediterranean Sea, through Messinian straits and/or Sicilian Channel, as previously suggested by Frantzis *et al.* (2011). It is important to note that 77% of Mediterranean samples (80 of 104) were males, whose mobility is likely different from that of females, which have a higher degree of site fidelity. Male sperm whale home range can be huge and expands with age and growth (Best, 1979; Whitehead and Weilgart, 2000). In the oceans, thanks to tags deployed by whalers, the wider record of males' movement has been of 4300 km longitudinally and 7400 km latitudinally (Mitchell 1975; Ivashin 1967). In western Mediterranean Sea, evidence of males photoID recaptures, identified in Gibraltar Straits, have been occurred in Alboran Sea, Balearic island and Ligurian Sea, with the longest movement of about 1500 km (Carpinelli *et al.*, 2014). Comparison of the photo-identification catalogue from Ligurian Sea and Tyrrenian Sea (Ischia island) revealed recaptures of three individuals (all immature males), transfers from north to south and vice versa, being recorded in both seas 1–2 months apart in the same summer, at distance of 600 km apart (Mussi *et al.* 2014). Finally, photo id recaptures of 2 stranded individuals between Ligurian Sea and Adriatic Sea, and of 1 alive individual between Ligurian Sea and Hellenic Trench, were the main evidences of inter-basin

movements, with the longest distance reported the Mediterranean, about 1800-2100 km (Frantzis *et al.*, 2011). The results of the present study are consistent with these findings from photoID methods, since they confirm the wide range of sperm whale movements within both Mediterranean basins and reject the presence of isolated area and population.

Despite no East and West clusters separation has been found – which would have been justified by allopatry - all the described analyses revealed the presence of a second Mediterranean cluster, well separated from the first one (see fig. 4.2.1-2-3 in population structure results). This latter was named MedB and has been found in sympatry with MedA – potentially it was unexpected. All analyses on population structure verified the divergence of this cluster from both MedA and Atl clusters. Pairwise Fst comparisons were significant (P-value=0.000) and respectively 0.01037 and 0.02512, suggesting a low but significant divergence of this cluster – closer to MedA than to Atl. The geneflow rates values between MedA and MedB clusters, about 1-2%, and between Atl and MedB, about 3%, suggest a low inward geneflow from the Atlantic area towards Mediterranean. For understanding this divergence and gene flow rates values, ancestry results show that this cluster has derived from the two neighbour populations, MedA and Atl: the degree of hybridisation in MedB population reflects the admixture between the Atlantic and Mediterranean lineages (see fig. 4.7.1-4). Indeed, **admixture** is the formation of a new genetic combination through hybridization of genetically distinct stocks/populations (see Allendorf *et al.*, 2009). The study of the causes and consequences of natural hybridization in hybrid zones - areas where genetically distinct stocks/populations meet and mate, generating some offspring with mixed ancestry (Harrison, 1993) - offers important information to evaluate and quantify the effects of gene flow and natural selection in natural populations (Mullen *et al.*, 2009). The sampling location of individuals belonging to MedB have been in the western side of Ligurian Sea, between 41.262° and 43.727° N and 5.244° and 8.280° E. These “MedB” samples represent the 63% (38 of 60 samples) of individuals sampled in Ligurian Sea. The left

27% (22 of 60 samples) belong to MedA cluster. This important finding suggests the presence of two different populations in the same area, without any geographical boundary, thus in sympatry. To support this divergence, the sampling year shows a clear separation. All samples collected in Ligurian Sea are from skin biopsies of free-ranging individuals: in detail, samples of MedB have been collected between year 2010 and 2013 and sample of MedA have been collected between years 2016 and 2018 (see fig. 4.3.1 in the results chapter). Therefore, we can speculate that the Ligurian Sea is used by these two populations in different years frame, even if more proofs are needed. Several studies assessed sperm whale movements from Ligurian Sea to Balearic Islands (see Rendel *et al.*, 2014), Gibraltar Strait (Carpinelli *et al.*, 2014), Tyrrenian Sea (Alessi *et al.*, 2014; Mussi *et al.*, 2014). All these evidence on wide and long movements may explain the absence of those Medb individuals in any sampling year. However, it is needed to underline that the sampling effort has not been homogeneous for all chosen areas. There is a strong sampling bias in Ligurian Sea and the occurrence of MedB cluster **just within** Ligurian Sea could be a sampling artifact. For population genetic studies, getting sperm whale samples, with DNA of good quality and quantity is a hard challenge: sampling effort is high and times to get enough samples are very long. On 8 Mediterranean partners that join this project, only one had the opportunity to collect sperm whale samples since 2010 to 2018, during every sampling season, and in a specific area (from Ligurian sea to South West of France). All the other partners provided samples from rare stranding events and from previous sampling campaigns. Further studies, using a higher number of samples from all analysed areas in this project and from unexplored and unsampled areas (such as North Africa), may help in finding more individuals from MedB cluster in other areas and clarify its dynamic and home range.

Further analyses, such as ABC (Cornuet *et al.*, 2014) not available at the moment, are needed and will clarify the origin and the demographic dynamic of the MedB cluster.

5.2 Environmental factors and genetic structure

Several studies on some marine species have confirmed hypothesis that environmental factors can shape genetic structure (Banks *et al.*, 2007). However, for sperm whales, as expected, RDA analyses did not support the role of SST, Salinity and ChlA as a factor in building genetic structure. Several authors have previously noticed a link between sperm whale density and primary production (Jaquet *et al.* 1995, Jaquet & Whitehead 1996). For example, Oliveira *et al.* (2016) showed that sperm whales foraging in the "oligotrophic" region around the Azores make shorter search and foraging phases and captured less prey per dive. However, these facts may be counterbalanced by the consumption of more nutritious prey (Clarke 1956; 1993). Similarly, sperm whales may stay in lower productive areas, such as the Mediterranean Sea due to the presence of prey patches associated with physiographic factors. These areas have lower total productivity values in relation to coastal areas but may represent a niche that still offers enough food supplies to live, breed and raise calves. In Mediterranean Sea, the regular preference of sperm whales in area as the Hellenic Trench may looks like an ecological paradox because of its low productivity. Probably, meso- and bathypelagic squids are not linked spatially to the primary production in surface waters (see Call *et al.*, 2013). This is in contrast with observations in other parts of the world ocean, where a link between sperm whale distribution and sea surface chlorophyll could be established (Jaquet, 1996; Jaquet *et al.*, 1996; O'Hern and Biggs, 2009). We propose that, sperm whale genetic structure is the end product of environmental pressures, social structure and environmental factors, but we cannot identify specific mechanisms by which the chosen parameters interact with sperm whale ecology in order to produce the observed pattern of genetic structure. The high mobility of this species gives them the opportunity to explore different areas and to have a wide home range, so that sperm whales can exploit different areas and cover huge distances in short time (Whitehead *et al.* 2003).

5.3 Sex classes occurrence in Ligurian Sea

To provide results on the sex classes segregation within the northern point in the Western basin, the sex of individuals from this area has been assessed genetically. The used protocol described in Berubè and Palsboll (1996) did not provide results straight forward: several tests, not suggested in the protocol, have been needed in order to get the amplified product. Above all, the two set of primers never worked if used at the same time. A sort of primer competition happened, which is not described in the paper. The PCR reactions needed to be run separately and then the final products have been combined in order to assess the sex visually on agarose gel.

Analysing the results, in the Ligurian stratum, composed of 60 samples, 45 individuals were males and 15 individuals were females with sex ratio 1:0.33 (respectively 32 males and 6 females from MedB, 13 males and 9 females from MedA). Compared to a sex ratio 1:1, our results give evidence of higher presence of males to females on this area, as suggested by Drouot *et al.* (2004, 2007) but not exclusive, as demonstrated by sightings of several social units in these sampling campaigns (Denis Ody and Celine Tardy, WWF-France – personal communication) and previous reports (see Moulins and Würtz, 2005; Calogero *et al.,* 2019). Furthermore, kinship results provided evidence of close kin only within groups from the same sampling event (see next paragraph). These results indicate that female groups and some social units also visit the area, which is not exclusive used by males. Probably females habit some restricted area and are less detectable than males. We can speculate that females need to use feeding area in the northern point of the Mediterranean as males do, to reduce sex classes competitions for preys, derived by the reduce latitudinal range of our basin (see Rendell and Frantzis, 2016) and also for seasonality of some preys, as found in California currents (Litz *et al.*, 2011). However, the northern point of Mediterranean is on 44°N of Latitude. In the ocean, female can rich the 50° N (Whitehead, 2018). We need to underline that most of monitoring project do not cover wintertime, and most of the information we have, derive from summertime surveys. Analyses on data from

autumn and wintertime would lead to a clearer picture about habitat use and age/sex classes of sperm whale in the western Mediterranean.

5.4 Kin Assessment

Among cetaceans, killer whales and sperm whales are typically recognized for having the strongest social ties, an intermediate level of dispersion and medium group size compared with other odontocetes (see Whitehead, 2017). The sperm whale is a species that shows evidence for matrifocal social structure (Whitehead, 2003). It is well known that hunting on cephalopod prey and with deep diving behaviour, female sperm whales create long-term bonds in their social structure that led to the communal care of young (Whitehead, 1996). Several studies performed through observation of recognized individuals confirmed long-term relationships within matrilineal groups (Christal and Whitehead, 2001), though associating pairs were not necessarily close kin (Richard *et al.*, 1996). In this framework, samples provided for this project have been collected by different teams, in different areas, different years and through not coordinated sampling strategies. Most of the samples were not collected within social units/groups. Despite that, preliminary analyses on social structure have been done, giving the first genetic relatedness results within Mediterranean Sea. It is important to underline that to perform a detailed population structure analyses, samples must come from individuals which are not closely related. Our kinship analyses revealed a general trend of unrelatedness, both among and within populations (pi_hat<0.125, see violin plot figures 4.9.1). No evidence of close kin relationships was found between Atlantic and Mediterranean populations (see violin plots in fig. 4.9.1 and grm matrix in fig. 4.9.4). This may be explained by the low gene flow rates from Atlantic. Some low kin degree, mainly from third to second degree (0.125<pi_hat<0.25), have been found between the two Mediterranean populations. Within each population, some evidence of close kin individuals can be seen only considering sampling areas location (see fig 4.9.3). Particularly, pi_hat≈0.5 values - corresponding to

parent/offspring and full siblings – can be found among individuals from Greece and from stranding events in Adriatic Sea, and with a single individual sampled in the southern side of Messinian straits. These results give some evidence of the presence of kin structure in the central and in the eastern Mediterranean Sea, where all kind of social groups can be found as suggested by Frantzis *et al.* (2014). Another interesting finding is that within groups of Ligurian Sea (both from MedA and MedB clusters) and within groups of each mass stranding events in Adriatic Sea, both kin and non-kin members have been found, as suggested in earlier studies, conducted outside of Mediterranean Sea (see Richard *et al.* 1996; Mesnick 2001; Gero *et al.* 2008; Pinela *et al.* 2009).

In conclusion, no close kin cluster has been detected for each population, suggesting the wide home range of sperm whale in the whole Mediterranean basin. Our results are consistent with those ones from Gulf of Mexico showed in Ortega *et al.* (2012), where the overall average kinship between individuals within groups was not greater than the average kinship among individuals from different groups, and close to zero. Furthermore, most of our samples were from males (73% in MedA and 84%in MedB) which are known to have a higher dispersal behaviour than females, and therefore the absence of high relatedness rates in our results could be expected. Considering that the main goal of this study is to find evidence of population structure within the Mediterranean, detailed analyses on social structure coupling data from genetic, observations and photoID are not been conducted yet. Further analyses at group level and on samples social unite will be done in order to compare this aspect with areas such as Gulf of Mexico (see Ortega *et al.,* 2012).

5.5 Demography

In wildlife populations, assessing the effective population size is important in order to predict inbreeding rate and loss of genetic diversity. Since the census population size is, for some species, usually available, it is hard to define the ratio of effective/actual population size (Ne/Nc) (Frankham, 1995). For cetacean species, census population size is a complex point and long-term studies are needed to have confident results on that. Above all for species as sperm whale, which spend 70% of its time in "feeding mode" below the surface (see Diving and feeding behaviour paragraph in the Introduction chapter), actual population size assessment is a hard challenge to achieve through only observational studies. Up to now, sperm whale worldwide estimation suggests a current population of about 360,000 animals reduced from a pre-whaling population of about 1.200.000 whales (Whitehead, 2002).

All molecular demographic published analyses, performed through PSMC, suggested a general worldwide population decreasing since the Pliocene/Pleistocene boundary, followed be an increasing through last interglacial period (Eemian, 130.000-115.000 years ago), and then a marked bottleneck during last glacial period (80.000-20.000 years ago), followed by a new expansion at the end of last glacial maximum (see Morin *et al.*, 2018; Fan *et al.*, 2019). Although timing estimates and Ne values are approximate, the pattern suggested by our results from stairway plot for Atlantic population are partially consistent with actual knowledge. It has been suggested that worldwide sperm whale expansion started by a maternal Pacific Ocean lineage between 100.000 and 80.000 years ago (see Alexander *et al.*, 2017 and Morin *et al.*, 2018). Assuming the colonization of Atlantic around that time, our data support a following marked bottleneck, overlapped to last glacial period (from 115.000 to 11.700 years ago). This is consistent with the idea of an historical event affecting all populations in the oceans, as suggested in Lyrholm *et al.* (1996), but also in Alexander *et al.* (2017). Indeed, the late Pleistocene was a period of numerous extinctions for large mammals, because of combined effect of climate change and hunting (Lorenzen *et al.*, 2011). Those species that

survived experienced demographic bottlenecks, local extinctions and phylogeographic shifts (Stiller *et al.* 2010). For marine environment, episodes of ocean cooling/warming seem to have influenced the distribution and abundance of many cetacean species and such events could have led to restricted home ranges (Gaskin 1982). That was the possible scenario of sperm whale, whose suitable habitat for females was reduced up to 50% (see Morin *et al.*, 2018). Cold regions at high latitude were covered by ice, and sperm whale males, which usually feed in these regions, occupied the same home range as females and young groups - which normally do not range into cold waters (Best, 1979). This change of age/sex classes distribution and overlap could have led to a competition for resources and to a marked reduction of food availability. Our results show an expansion occurred between 30.000 and 15.000 years ago, consistent with the end of last glacial maximum. This latter result is also concordant with previous PSMC analyses of sperm whales (see molecular ecology paragraph in the Introduction, section 1.14). The recent expansion at the end of LGM probably followed the prey expansion. Indeed, the giant squid (*Architeuthis* spp.), one of sperm whale's prey in the ocean, shows extremely low mitogenomic diversity and signatures of demographic/range expansions associated with the LGM (Winkelmann *et al.* 2013), seen also in *Dosidicus gigas* (Ibanez *et al.* 2011) and *Doryteuthis gahi* (Ibanez & Poulin 2014). Further support of these finding come from other deep divers, such as the Gray's beaked whale and pilot whales, which showed similar expansion pattern (see Oremus *et al.*, 2009; Thompson *et al.*, 2016).

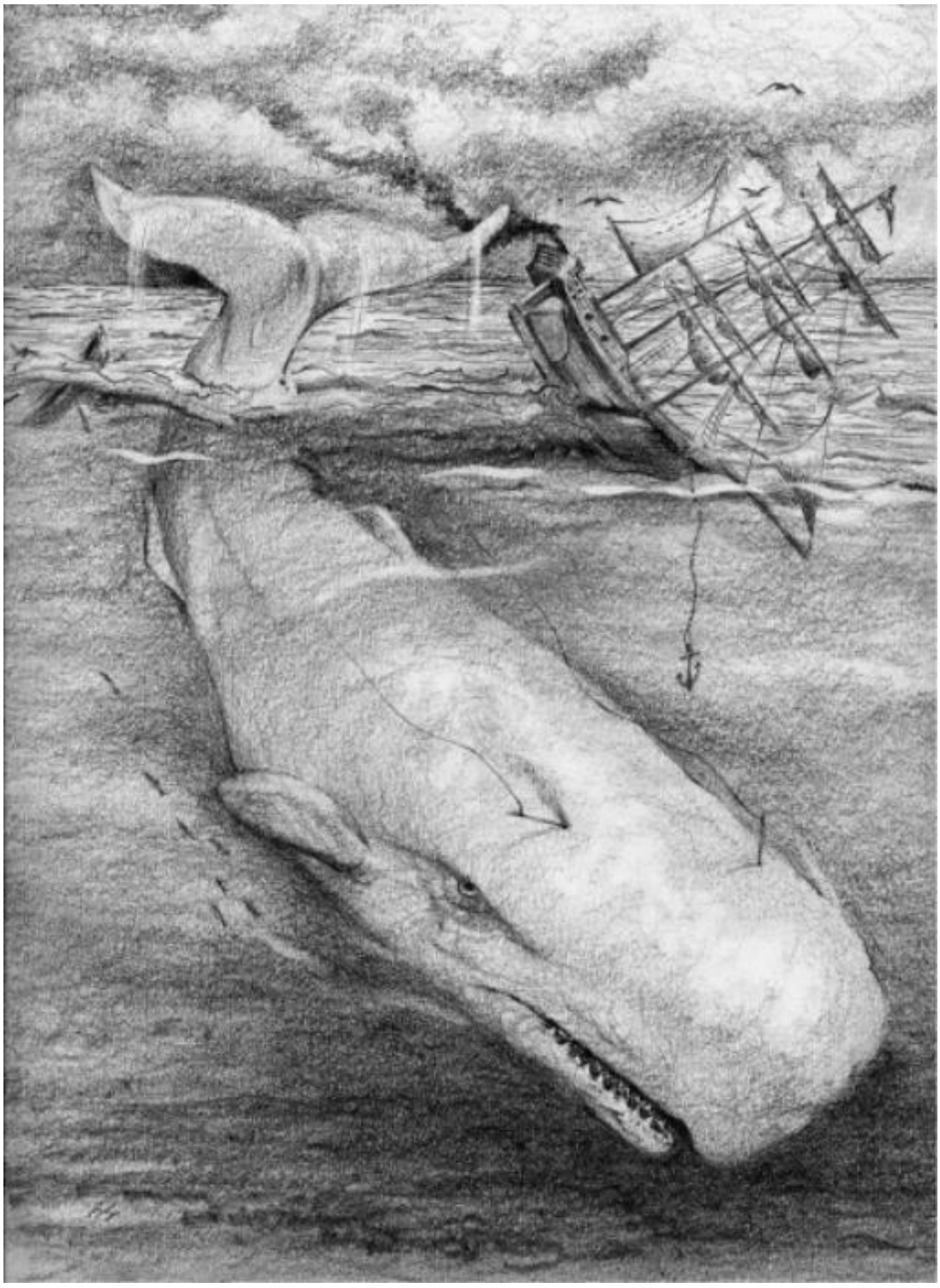
In the context of last glacial period, Mediterranean Sea probably played a key role as **refuge** for several marine species (see Patarnello *et al.* 2007). During Pleistocene glaciations, sea level was reduced up to 150 m (Lambeck & Purcell, 2005), changing oceanographic features that created isolated refugia and geographical barriers to gene flow, followed by post-glacial dispersal and expansion (Xue *et al.*, 2014). Previous studies have suggested that Mediterranean biodiversity is the result of endemism from glacial refugia (see Patarnello *et al.* 2007). This area might have also been a sink for many Atlantic

species during and post-LGM. Our results – for MedA population, in which the most ancient section of stairway plot reflects the found Atlantic pattern - suggest a scenario whereby the population in the Mediterranean was established recently as founder population at the end of LGM. Our results are therefore consistent with Morin *et al.* (2018) who assessed the divergence time of Mediterranean lineages to 20.000 years ago. The reduction of habitat suitable for sperm whale in Atlantic Ocean during last glacial period could have driven some sperm whale to colonize Mediterranean Sea. This dynamic is difficult to clarify and confirm. During LGM, changes in the pattern of deep-water availability in the western Mediterranean were moderate to substantial from western to eastern basin (Mikolajewicz *et al.*, 2011). In the western basin, sperm whale could have found suitable habitat to colonize and feed on deep squids, which probably weren't affected by ice age pressures (see Winkelman *et al.*, 2011), providing enough prey to support their metabolic demand were present.

However, despite the discussed results are not in contrast with actual knowledge, there are some limits in the site frequency spectrum methodology, as described in Patton *et al.* (2019). This latter gave evidence that methods reliant on the sequentially Markovian coalescent (as PSMC) are most reliable between ≈300 and 100.000 generations before present, whereas methods exclusively reliant on the site-frequency spectrum (as stairway plot) are most reliable between the present and 30 generations before present. In this framework, analysing our stairway plot within last 30 generations, the population dynamic within last 1.000 years can be estimated. In Atlantic Ocean, a general reduction trend can be seen, which probably reflects the high depletion rate of whaling time (see fig. 4.10.1). Ne values range from 600 to 10.000, with mean value of Ne≈4000 sexually mature individuals. Considering that species with social structure and/or strong bias in reproductive success, such as sperm whale, Ne/Nc ratio can be 0.1 or lower (Frankham, 1995), we can estimate approximately Nc≈80.000 in Atlantic Ocean. Unfortunately, our results on N cannot be compared with results from observation studies, no

such data exist. Furthermore, PSMC results gave results till 10.000 years ago and do not give recent Ne estimation. Therefore, our estimates need to be carefully considered and supported by further analyses with different methods and approaches.

In the Mediterranean, a reduction trend can be seen as well (see fig 4.10.2). Our Ne estimates give values ranging from 400 to 4000 and a mean of Ne≈1000-2000 sexually mature individuals, and lower than Atlantic Ocean, as we expected. This value is concordant with *IUCN-Red list* assessment (see Di Sciara, 2017; 2012), which classified Mediterranean sperm whale population as Endangered, thus with less than 2500 sexually mature individuals. It is also compatible with the current estimations for the western and eastern Mediterranean basins (Rendell and Frantzis, 2016; Lewis *et al.* 2007; Frantzis *et al.* 2019). However, here is quite hard to give estimates on actual population size (Nc), because of several lack of knowledge about social structure in the Mediterranean Sea – except few areas – and the absence of estimates on calf and juvenile mortality (see Frantzis *et al.*, 2014), and considering that here are no predators as in the open ocean (see Praca and Gannier, 2008). It is well known that sperm whales in Mediterranean are not directly threatened by fishery and are not facing prey depletion by human: the main prey of Mediterranean sperm whales is *H. bonnellii*, which is not a target of fishery activity. However, Mazzariol *et al.* (2011) found several hooks within stomach content analyses of stranded individuals. This finding cannot exclude the feeding activity of sperm whale along fishing lines as described in other oceanic regions (i.e. Jacobsen *et al.*, 2010). At the same time, fishery activity seems to have another impact on sperm whales through entanglement in fishing nets. Ghost nets, ship collision and ingestion of plastic debris are the three main impacts that are seriously threatening this endangered and small population.



6 Conclusion and Perspectives

Roger Payne said: "There is a message coming from the ocean to us, from the whales directly. What this message says is: it is possible to own a brain as complex as our own without destroying our world. What we have to learn from this message is very simple. If what we do diminish the ability of our planet to support life, then we don't have to do it! Or we have no future. Modern whales, for all their 20 million years, what is 19 million years more than us, have succeeded in living on our planet without destroying it. We could do just the same!"

This project has been run in order to get important results and clarify population structure and dynamics of sperm whale endangered population within Mediterranean Sea. The established network among researchers from different six different countries gave me the possibility to get many samples, already available from previous research projects and avoid new pressures on sperm whale with biopsy sampling collection. Up to know we have the biggest dataset of extracted DNA from the Mediterranean Sea.

The use of SNPs has revealed to be fundamental in case of populations with low genetic diversity and restricted home range, as sperm whales in the Mediterranean.

Our results have showed that the Sicilian waters are not barriers, but corridors for the gene flow and therefore we can assume that eastern basin can avoid a loss of genetic diversity and reduce the risk of inbreeding phenomena. These are important data considering that sperm whales face several

anthropogenic pressures in the Hellenic Trench, which is probably the most important if not unique breeding area for this species in the eastern basin.

The discovery of a second Mediterranean cluster, composed of hybrid population between Atlantic and Mediterranean needs to be supported by further analyses, which can clarify its historical dynamics.

The described pattern of recent geneflow through Gibraltar Straits confirm a movement of some individuals from Atlantic areas which can provide an inward gene flow to avoid genetic diversity reduction in the whole basin.

The detailed mechanism that drives the development of genetic structure requires future studies: in particular we need more data and information on the historical expansion events, in order to clarify how the two populations within the Mediterranean Sea have developed and diverged from the Atlantic population.

Further analyses using different bioinformatic methods will be performed in order to support all the described findings up to know. More data from unsampled areas (such as Turkey and North Africa regions) should be added.

A key point is to promote a next project on wide scale, through photoID methods, in order to assess individual pattern of connectivity and assemble these results with the genetic. These new data are needed to provide scientific support to conservation action. Considering the increasing impact of human activities on marine life, it is not possible to guarantee that sperm whales and other top predators will persist in the Mediterranean Sea. Their role is of great importance in the balance of marine ecosystems and their monitoring and conservation should be one of key objectives within international managers and stakeholders' actions.

Heracles battles the Trojan Cetus, which is a representation of a sperm whale

Stavros S. Niarchos Collection, Athens

ca 530 - 520 B.C

7 Appendix

Table S1: List of samples after filtering and thinning

NAME	Nr	SAMPLING AREA	AREA COLOURS	POP CLUSTER	CLUSTER COLOURS
A153	1	Azores	blue2	Atl	blue2
A155	2	Azores	blue2	Atl	blue2
A176	3	Azores	blue2	Atl	blue2
A192	4	Azores	blue2	Atl	blue2
A195	5	Azores	blue2	Atl	blue2
A223	6	Azores	blue2	Atl	blue2
A231	8	Azores	blue2	Atl	blue2
A232	9	Azores	blue2	Atl	blue2
A236	10	Azores	blue2	Atl	blue2
A237	11	Azores	blue2	Atl	blue2
A241	12	Azores	blue2	Atl	blue2
B11	14	Balearic	darkorange3	MedA	blue2
B13	15	Balearic	darkorange3	MedA	blue2
B15	16	Balearic	darkorange3	MedA	blue2
B16	17	Balearic	darkorange3	MedA	blue2
B17	18	Balearic	darkorange3	MedA	blue2
B18	19	Balearic	darkorange3	MedA	blue2
B9	20	Balearic	darkorange3	MedA	blue2
C10	21	Canarias	deeppink4	Atl	blue2
C11	22	Canarias	deeppink4	Atl	blue2
C13	23	Canarias	deeppink4	Atl	blue2
C14	24	Canarias	deeppink4	Atl	blue2
C15	25	Canarias	deeppink4	Atl	blue2
C2	26	Canarias	deeppink4	Atl	blue2
C3	27	Canarias	deeppink4	Atl	blue2
C7	29	Canarias	deeppink4	Atl	blue2
C8	30	Canarias	deeppink4	Atl	blue2
C9	31	Canarias	deeppink4	Atl	blue2
F1	32	Ligurian	red2	MedB	blue2

F11	33	Ligurian	red2	MedB	blue2
F12	34	Ligurian	red2	MedA	blue2
F13	35	Ligurian	red2	MedB	blue2
F14	36	Ligurian	red2	MedB	blue2
F17	37	Ligurian	red2	MedB	blue2
F18	38	Ligurian	red2	MedB	blue2
F19	39	Ligurian	red2	MedB	blue2
F2	40	Ligurian	red2	MedB	blue2
F20	41	Ligurian	red2	MedB	blue2
F22	43	Ligurian	red2	MedB	green
F24	44	Ligurian	red2	MedB	green
F25	45	Ligurian	red2	MedB	green
F26	46	Ligurian	red2	MedA	green
F27	47	Ligurian	red2	MedB	green
F28	48	Ligurian	red2	MedB	green
F29	49	Ligurian	red2	MedA	green
F3	50	Ligurian	red2	MedB	green
F30	51	Ligurian	red2	MedB	green
F32	52	Ligurian	red2	MedB	green
F33	53	Ligurian	red2	MedB	green
F34	54	Ligurian	red2	MedB	green
F35	55	Ligurian	red2	MedB	green
F36	56	Ligurian	red2	MedB	green
F39	57	Ligurian	red2	MedB	green
F4	58	Ligurian	red2	MedB	green
F40	59	Ligurian	red2	MedB	green
F41	60	Ligurian	red2	MedB	green
F42	61	Ligurian	red2	MedB	green
F43	62	Ligurian	red2	MedB	green
F45	63	Ligurian	red2	MedB	green
F46	64	Ligurian	red2	MedB	green
F47	65	Ligurian	red2	MedB	green
F48	66	Ligurian	red2	MedB	green
F49	67	Ligurian	red2	MedB	green
F5	68	Ligurian	red2	MedB	green
F50	69	Ligurian	red2	MedB	green
F52	70	Ligurian	red2	MedB	green
F53	71	Ligurian	red2	MedA	green
F54	72	Ligurian	red2	MedA	green
F55	73	Ligurian	red2	MedA	green
F56	74	Ligurian	red2	MedA	green
F57	75	Ligurian	red2	MedA	green

F58	76	Ligurian	red2	MedA	green
F59	77	Ligurian	red2	MedA	green
F6	78	Ligurian	red2	MedB	green
F60	79	Ligurian	red2	MedA	green
F61	80	Ligurian	red2	MedA	green
F62	81	Ligurian	red2	MedA	green
F63	82	Ligurian	red2	MedA	red
F67	83	Ligurian	red2	MedA	red
F68	84	Ligurian	red2	MedA	red
F69	85	Ligurian	red2	MedA	red
F7	86	Ligurian	red2	MedB	red
F70	87	Ligurian	red2	MedA	red
F71	88	Ligurian	red2	MedA	red
F72	89	Ligurian	red2	MedA	red
F73	90	Ligurian	red2	MedA	red
F74	91	Ligurian	red2	MedA	red
F8	92	Ligurian	red2	MedB	red
G16	94	Greece	forestgreen	MedA	red
G17	95	Greece	forestgreen	MedA	red
G1B	96	Greece	forestgreen	MedA	red
G3	98	Greece	forestgreen	MedA	red
G30	99	Greece	forestgreen	MedA	red
G33	100	Greece	forestgreen	MedA	red
G65	103	Greece	forestgreen	MedA	red
G68	104	Greece	forestgreen	MedA	red
G72	107	Greece	forestgreen	MedA	red
G85	108	Greece	forestgreen	MedA	red
G86	109	Greece	forestgreen	MedA	red
G88	110	Greece	forestgreen	MedA	red
G93	114	Greece	forestgreen	MedA	red
I10	115	Adriatic	orange	MedA	red
I12	116	Adriatic	orange	MedA	red
I13	117	Adriatic	orange	MedA	red
I14	118	Adriatic	orange	MedA	red
I15	119	Adriatic	orange	MedA	red
I16	120	Adriatic	orange	MedA	red
I17	121	Adriatic	orange	MedA	red
I18	122	Adriatic	orange	MedA	red
I21	124	Adriatic	orange	MedA	red
I22	125	Adriatic	orange	MedA	red
I25	126	Adriatic	orange	MedA	red
I6	131	Tyrrenian	chocolate4	MedA	red

I7	132	Tyrrenian	chocolate4	MedA	red
I8	133	Tyrrenian	chocolate4	MedA	red
M1	134	Madeira	seagreen4	Atl	red
M10	135	Madeira	seagreen4	Atl	red
M11	136	Madeira	seagreen4	Atl	red
M13	137	Madeira	seagreen4	Atl	red
M14	138	Madeira	seagreen4	Atl	red
M2	139	Madeira	seagreen4	Atl	red
M3	140	Madeira	seagreen4	Atl	red
M4	141	Madeira	seagreen4	Atl	red
M5	142	Madeira	seagreen4	Atl	red
M6	143	Madeira	seagreen4	Atl	red
M7	144	Madeira	seagreen4	Atl	red
M8	145	Madeira	seagreen4	Atl	red
M9	146	Madeira	seagreen4	Atl	red
NS2	147	NorthSpain	grey1	Atl	red
NS3	148	NorthSpain	grey1	Atl	red
NS4	149	NorthSpain	grey1	Atl	red
NS5	150	NorthSpain	grey1	Atl	red
S12	151	Gibraltar Straits	darkturquoise	MedA	red
S14	152	Gibraltar Straits	darkturquoise	MedA	red
S15	153	Gibraltar Straits	darkturquoise	MedA	red
S16	154	Gibraltar Straits	darkturquoise	MedA	red
S18	155	Gibraltar Straits	darkturquoise	MedA	red
S2	156	Gibraltar Straits	darkturquoise	MedA	red
S5	157	Gibraltar Straits	darkturquoise	MedA	red
S7	158	Gibraltar Straits	darkturquoise	MedA	red
S8	159	Gibraltar Straits	darkturquoise	MedA	red
S9	160	Gibraltar Straits	darkturquoise	MedA	red

Table S3. Sex of individuals

Name	Sex	Name	Sex	Name	Sex	Name	Sex	Name	Sex
A153	F	M5	F	F39	M	F54	F	G88	M
A155	M	M6	F	F4	M	F55	F	G93	M
A176	F	M7	F	F40	M	F56	F	I6	F
A192	M	M8	F	F41	M	F57	F	I7	M
A195	F	M9	F	F42	M	F58	F	I8	M
A223	F	NS2	M	F43	M	F59	M	I10	M
A231	F	NS3	F	F45	M	F60	F	I12	M
A232	F	NS4	M	F46	M	F61	F	I13	M
A236	M	NS5	F	F52	M	F62	F	I14	M
A237	F	F1	M	F6	F	F63	M	I15	M
A241	F	F11	F	F7	F	F67	M	I16	M
C10	M	F13	F	F8	F	F68	M	I17	M
C11	F	F14	M	F30	M	F69	M	I18	F
C13	F	F17	M	B11	M	F70	M	I21	M
C14	F	F18	M	B13	M	F71	M	I22	F
C15	M	F19	M	B15	M	F72	M	I25	F
C2	M	F2	M	B16	M	F73	M	S2	M
C3	F	F20	M	B17	M	F74	M	S5	M
C7	F	F22	M	B18	M	G16	F	S7	M
C8	F	F24	M	B9	F	G17	F	S8	M
C9	F	F25	M	F12	F	G1B	M	S9	M
M1	M	F27	M	F26	M	G3	M	S12	M
M10	F	F28	M	F29	M	G30	M	S14	M
M11	F	F3	M	F47	M	G33	M	S15	M
M13	F	F32	M	F48	M	G65	F	S16	M
M14	M	F33	M	F49	M	G68	M	S18	M
M2	F	F34	M	F5	F	G72	M		
M3	F	F35	M	F50	M	G85	M		
M4	M	F36	M	F53	M	G86	F		

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